0090-9556/09/3712-2323–2329$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 37, No. 12
28605/3536343
DMD 37:2323–2329, 2009
Printed in U.S.A.

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

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Received May 27, 2009; accepted September 17, 2009

ABSTRACT:

We tested the hypothesis that primary cultures of human hepatocytes could predict potential drug interactions with methadone and buprenorphine. Hepatocytes (five donors) were preincubated with dimethyl sulfoxide (DMSO) (vehicle), rifampin, or nelfinavir before incubation with methadone or buprenorphine. Culture media (0–60 min) was analyzed by liquid chromatography-tandem mass spectrometry for *R*- and *S*-methadone and *R*- and *S*-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) or for buprenorphine, norbuprenorphine, and their glucuronides [buprenorphine-3-glucuronide (B-3-G) and norbuprenorphine-3-glucuronide (N-3-G)]. *R*- and *S*-EDDP were detected in three of five, four of five, and five of five media from cells pretreated with DMSO, nelfinavir, and rifampin. *R*-EDDP increased 3.1- and 26.5-fold, and *S*-EDDP increased 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 of the buprenorphine loss from culture media; it was not significantly affected by either pretreatment. Norbuprenorphine and N-3-G together were detected in three of five, four of five, and five of five donors pretreated with DMSO, nelfinavir and rifampin, and norbuprenorphine in one of five, one of five, and two of five donors. Although there was a trend for norbuprenorphine (2.8- and 4.9-fold) and N-3-G (1.7- and 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 glucuronidation; *N*-dealkylation predominated in small intestine and 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 the combined effects of hepatic and small intestinal metabolism are probably involved.

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

Many of these comedications 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 non-nucleoside 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 (Kreek et al., 1976; Kharasch et al., 2004); this induction was also associated with withdrawal syndromes (Kreek et al., 1976). More recently, studies on the interaction of buprenorphine with antiretroviral agents have been conducted. Efa-

This work was supported in part by the National Institutes of Health National Institute on Drug Abuse [Grant RO1-DA10100]; and the National Institutes of Health National Institute of General Medical Sciences [Grant GM066411]. 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]. For projects unrelated to the current study, D.E.M. has received research funding and consultant fees from Reckitt Benckiser, the manufacturer of buprenorphine.

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

doi:10.1124/dmd.109.028605.

ABBREVIATIONS: AUC, area under the time-concentration curve; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; P450, cytochrome P450; UGT, UDP-glucuronosyl transferase; HLM, human liver microsomes; HSIM, human small intestine microsomes; UDPGA, uridine 5′-diphosphoglucuronic acid; DMSO, dimethylsulfoxide; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; QC, quality control sample; AUIC, area under the incubation time-concentration curve.

virenz 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, whereas nelfinavir and the lopinavir/ritonavir combination had minimal effect on buprenorphine pharmacokinetics, none of which 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 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-diphenylpyrrolidine (EDDP), which may be further *N*-demethylated to 2-ethyl-5-methyl-3,3-diphenylpyrroline. These metabolites retain the *R*- and *S*-enantiomeric relationship. Although 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, making determination of their contribution to clearance of the parent drug hard to determine. The *N*-demethylation of methadone has been attributed to CYP3A4, CYP2B6, CYP2D6, and CYP2C19 (Iribarne et al., 1996; Moody et al., 1997; Kharasch et al., 2004). 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 (Picard et al., 2005; Chang et al., 2006). 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 buprenorphine has not been determined because of a lack of reference material. M1 is formed by both CYP3A4 and CYP2C8; M3 is formed primarily by CYP3A4 (Chang et al., 2006); they too are modulated by antiretroviral medications (Moody et al., 2009). The glucuronidation of buprenorphine is primarily carried out by UGT1A1 and UGT2B7 and that of norbuprenorphine by UGT1A1 and UGT1A3 (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 hepatocytes could be used to predict and to better define the in vivo interactions reported between methadone and buprenorphine with antiretroviral and antitubercular agents. In our investigation, we used the protease inhibitor, nelfinavir, as a model antiretroviral agent, because 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 (Kreek et al., 1976; Kharasch et al., 2004).

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 was 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 led us to propose the hypothesis that the small intestine plays an important role in the *N*-dealkylation of buprenorphine in humans.

Materials and Methods

Materials. Buprenorphine, d_4-buprenorphine, norbuprenorphine, d_3-norbuprenorphine, buprenorphine-3-β-D-glucuronide, norbuprenorphine-3-β-D-glucuronide, racemic methadone, racemic d_3-methadone, racemic EDDP perchlorate, and racemic d_3-EDDP perchlorate were purchased from Cerilliant Corporation (Round Rock, TX). Uridine 5′-diphosphoglucuronic acid (UDPGA), alamethicin, D-glucose 6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase, β-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 (end-capped C8, 500 mg/6 ml) were purchased from United Chemical Technologies, Inc. (Bristol, PA). Organic solvents used for extractions were high-performance liquid chromatography 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 five different donors were obtained from the University of Pittsburgh, through the Liver Tissue Cell Distribution System. Culture details have been reported previously (Olsavsky et al., 2007). In brief, hepatocytes were plated on collagen-coated six-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 Williams' E Medium supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 μM glutamine, 25 nM dexamethasone, 10 mM insulin, 30 mM linoleic acid, 1 mg/ml bovine serum albumin, 5 ng/ml selenious acid, and 5 μg/ml transferrin. Forty-eight hours after Matrigel additions, cells were exposed to either rifampicin (8.93 μg/ml), nelfinavir (2.0 μg/ml), or DMSO (vehicle control) for 96 h and then were treated with buprenorphine (2.5, 5, or 10 ng/ml), methadone (100, 300, 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 two wells/treatment arm were analyzed in parallel. During the hepatocyte culture period, media were 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. (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 being 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 of

protein/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 of methanol, and samples were quickly moved to −75°C storage until analysis.

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. (2008). Aliquots (100 μl) of hepatocyte culture media were fortified with d_3-methadone and d_3-EDDP as an 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 high-performance liquid chromatography system equipped with a Chiral-AGP column (50 × 2.0 mm, 5 μm; Analytical Sales and Services Inc., Pompton Plains, NJ). A Finnigan/Thermo (Thermo Fisher Scientific, Waltham, MA) Quest TSQ triple-stage quadrupole mass spectrometer was used for analysis. All analytical batches included two sets of calibrators ranging from 2.5 to 500 ng of enantiomer/ml, and duplicate quality control samples (QCs) at 5.0, 100, and 425 ng of 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. (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, and the eluates were dried and reconstituted in 0.1% formic acid in water-acetonitrile (98:2, v/v). Chromatographic separation was achieved using a Surveyor liquid chromatography system (Thermo Fisher Scientific) equipped with a YMC ODS-AQ 3 μ column (50 × 2 mm; Waters, Milford, MA). A TSQ-Quantum (Thermo Fisher Scientific) triple-stage quadrupole mass spectrometer was used for analysis. All analytical batches included two sets of calibrators ranging from 0.1 to 50 ng/ml, and duplicate QCs 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, area under the incubation time-concentration curve $(AUIC)_{0-60\ min}$ values were determined using the trapezoidal rule. For hepatocyte incubations the results from the five donors across the three treatment groups (DMSO controls, rifampin, and nelfinavir) were first compared using repeated-measures analysis of variance. If a significant difference was found at two-tailed $p < 0.05$, the Tukey post hoc test was used to determine differences between groups (two-tailed $p < 0.05$). GraphPad InStat (version 3.0; GraphPad Software Inc., San Diego, CA) was used for these determinations. Comparisons between *R*- and *S*-methadone utilization and *R*- and *S*-EDDP formation were made with Student's paired *t* test using Excel:Mac 2001 (Microsoft, Redmond, 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.

TABLE 1

Donor demographics

Donor	Age	Gender	Race	Clinical Information
	yr			
A	53	F	White	Resection, nonmalignant growth in liver
B	44	M	White	Resection, colon cancer with metastasis to liver
C	60	F	White	Resection, cholangiocarcinoma
D	69	F	White	Resection, hepatocellular carcinoma
E	49	M	White	Resection, metastatic colon cancer, chemotherapy

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 Fig. 1. The mean and individual derived $AUIC_{0-60\ min}$ values 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-min 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 three of five, four of five, and five of five 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 with vehicle controls in $AUIC_{0-60\ min}$ for *R*- and *S*-EDDP, respectively) than with either of the other treatment groups. The $AUIC_{0-60\ min}$ *R*- and *S*-EDDP productions after nelfinavir were 3.1- and 2.5-fold compared with 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 Fig. 2. The mean and individual derived $AUIC_{0-60\ min}$ values 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 $AUIC_{0-60\ min}$ (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 one of five, two of five, and one of five donors pretreated with DMSO, rifampin, and nelfinavir; norbuprenorphine-3-glucuronide in three of five, four of five, and four of five donors pretreated with DMSO, rifampin, and nelfinavir. Although norbuprenorphine production tended to increase relative to vehicle control with pretreatment with rifampin (4.9-fold increase in $AUIC_{0-60\ min}$) and nelfinavir (2.6-fold increase in $AUIC_{0-60\ min}$), these increases were not significant and were based on positive findings in two donors and one donor. For norbuprenorphine-3-glucuronide $AUIC_{0-60\ min}$, there was no significant change between treatment groups. If the combined formation of norbuprenorphine and norbuprenorphine-3-glucuronide is considered, the tendency for increased $AUIC_{0-60\ min}$ relative to vehicle control continued (1.35-fold with rifampin and 1.19-fold with nelfinavir), but these were also not

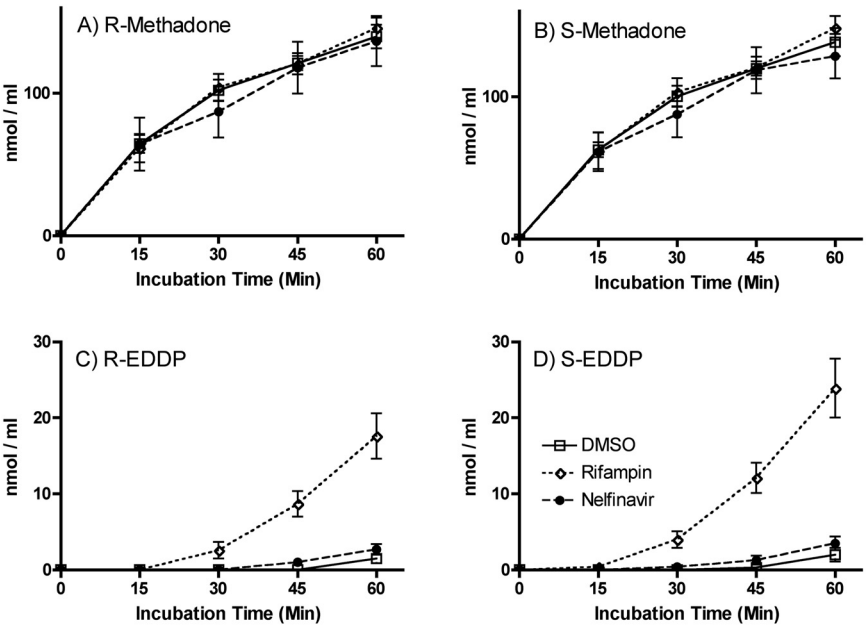

FIG. 1. Time course of *R*-methadone (A) and *S*-methadone (B) utilization and *R*-EDDP (C) and *S*-EDDP (D) formation in media from cultured human hepatocytes that had been preincubated with DMSO, rifampin, or nelfinavir and incubated with 300 ng/ml racemic methadone. Results are the mean of results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

TABLE 2

Area under the incubation-time curve for R-methadone and S-methadone 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

Treatment	Donor	Utilization		Production	
		R-Methadone	*S*-Methadone	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
		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
		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
		5.06 ± 2.04†	4.97 ± 1.73†	35.7 ± 25.9[a]†	50.5 ± 39.2[a]†
p (repeated-measures ANOVA)		0.861	0.678	0.0013	0.0003

ANOVA, analysis of variance.
* Significant difference ($p < 0.05$) between *R*- and *S*-enantiomers based on Student's paired *t* test.
† Mean ± S.D.
[a,b] Groups sharing the same letter are not significantly different ($p < 0.05$) based on Tukey post hoc tests when repeated-measures ANOVA showed significance ($p < 0.05$) between groups.

significant (data not shown). In general, the production of all three metabolites increased with increases in buprenorphine concentration, but there were some cases in which no norbuprenorphine 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 (Moody et al., 2002; Chang and Moody, 2005; Huang et al., 2006). 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 with both a source of NADPH and UDPGA along with the facilitator of glucuronidation, alamethicin (Fig. 3). Under these conditions, HLM produced all three metabolites with the following $AUIC_{0-60\ min}$ values: 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 $AUIC_{0-60\ min}$ (0.61) was still less than that of buprenorphine-3-glucuronide. HSIM were also active in metabolizing buprenorphine; the $AUIC_{0-60\ min}$ for buprenorphine utilization was 29% of that in HLM. HSIM produced both norbuprenorphine and buprenorphine-3-glucuronide with respective AUIC values 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

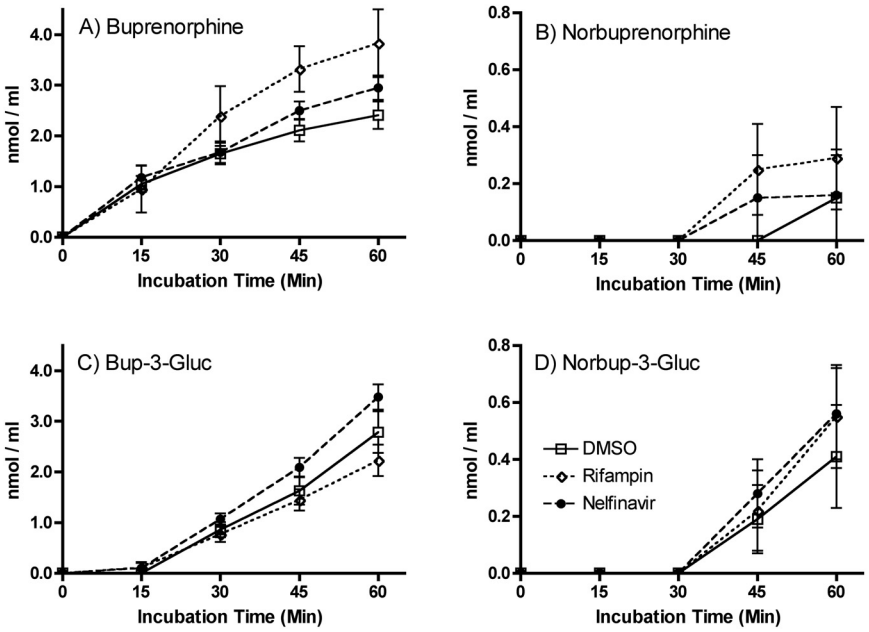

FIG. 2. Time course of buprenorphine (A) utilization and norbuprenorphine (B), buprenorphine-3-glucuronide (Bup-3-Gluc) (C), and norbuprenorphine-3-glucuronide (Norbup-3-Gluc) (D) formation in media from cultured human hepatocytes that had been preincubated with DMSO, rifampin, or nelfinavir and incubated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

TABLE 3

Area under the incubation-time curve for buprenorphine utilization and norbuprenorphine, buprenorphine-3-glucuronide, and nobuprenorphine-3-glucuronide formation in media from cultured hepatocytes treated with DMSO, rifampin, or nelfinavir and incubated with 5 ng/ml buprenorphine

Treatment	Donor	Utilization: Bup	Production		
			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
		90.2 ± 22.5[a]	1.2 ± 2.6[a]	58.2 ± 21.0[a]	5.4 ± 6.5[a]
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
		129 ± 58[a]	5.9 ± 8.3[a]	51.5 ± 19.5[a]	7.5 ± 7.4[a]
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
		102 ± 23[a]	3.4 ± 7.6[a]	75.2 ± 16.1[a]	8.4 ± 6.4[a]
p (repeated-measures ANOVA)		0.150	0.241	0.102	0.647

Bup, buprenorphine; Nor, norbuprenorphine; B-3-G, buprenorphine-3-glucuronide, N-3-G, nobuprenorphine-3-glucuronide; ANOVA, analysis of variance.
[a] Mean ± S.D.

systems, but the ratio of the $AUIC_{0-60\ min}$ of *N*-dealkylation to glucuronidation remained higher in HSIM (3.85) than in 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 glucocorticoid, 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 (Hoffmaster et al., 2004; Annaert and Brouwer, 2005), preservation of hepatocyte differentiation markers, hepatic nuclear receptors, and drug-metabolizing activities (Kern et al., 1997; Sidhu et al., 2004; Olsavsky et al., 2007; Page et al., 2007), and maintenance of cellular induction responses to phenobarbital, a sensitive indicator of a well differentiated hepatocyte (Sidhu et al., 2004; Olsavsky et al., 2007). 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

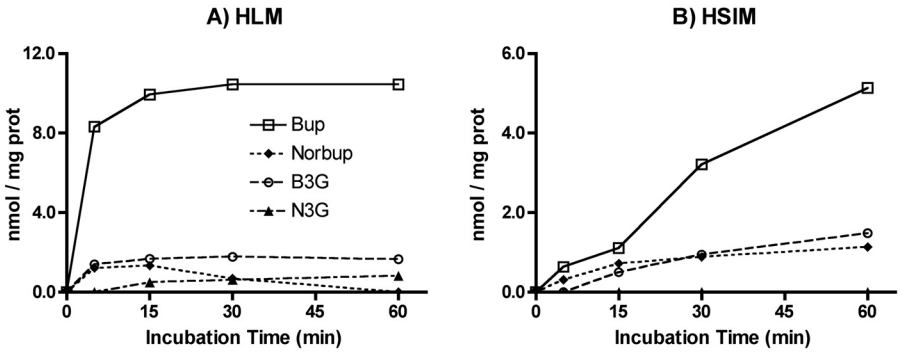

FIG. 3. Time course of buprenorphine (Bup) utilization and norbuprenorphine (Norbup), buprenorphine-3-glucuronide (B3G), and norbuprenorphine-3-glucuronide (N3G) formation in HLM (A) and HSIM (B) incubated with 5 ng/ml buprenorphine. Results are the mean of duplicate incubations.

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 (D. E. Moody and Y. Chang, unpublished observations), but nothing near the 10- to 30-fold observed here with hepatocytes. One can speculate that this excess could arise from a large contribution of nonmicrosomal metabolism and/or a time-dependent uptake of methadone into the hepatocytes. Although other metabolites of methadone have been observed, the formation of the methadols and ring hydroxylation products are consistent with cytochrome P450 (P450)-mediated reactions. Few reports on the involvement of drug transporters in methadone disposition are available beyond findings that it is not closely linked to P-glycoprotein genetics (Crettol et al., 2006) nor is its disposition affected 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 (Kreek et al., 1976; Kharasch et al., 2004). It is noteworthy that we detected a decrease in the ratio of *S*- to *R*-EDDP production, suggesting that CYP3A was induced preferentially over CYP2B6 after 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 three donors were quite responsive to the inductive effects of nelfinavir, the other two 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 result 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 that in the clinical interaction study in which 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. However, there was little indication of active *N*-dealkylation of buprenorphine occurring in the hepatocytes we studied. This finding 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 with buprenorphine-3-glucuronide in hepatocytes could be the result of glucuronide expression being better maintained in culture than P450s, 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 two-dimensional 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. (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 more than 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 P450s, 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 result points to other factors that may explain the paucity of norbuprenorphine formation detected in cultured hepatocytes.

Metabolites released from cultured hepatocytes reflect not only potential excretion into sinusoids and eventually into the systemic circulation but also excretion into the biliary canaliculi. 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, which in turn provides substrate for small intestinal metabolism. These findings are consistent with a hypothesis that buprenorphine is primarily glucuronidated in the liver, whereas it is primarily *N*-dealkylated in the small intestine. However, our current in vitro studies in HLM and HSIM 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 P450-mediated and glucuronidative metabolism (Mohutsky et al., 2006; Cubitt et al., 2009; Kilford et al., 2009). These have been limited to just monitoring of buprenorphine use and have used supratherapeutic concentrations of buprenorphine. We have now used such a combined system that included therapeutic concentrations of buprenorphine and monitored specific metabolite

formation, as well as buprenorphine use. Although 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. More recently, Cubitt et al. (2009) also demonstrated that HSIM can metabolize buprenorphine. However, their study used supratherapeutic 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 result is consistent with the growing knowledge of drug metabolism capabilities of the small intestine. In terms of the present study it seems 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 hepatocytes to the in vivo and microsomal metabolism of buprenorphine.

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