Contribution of the different UDP-glucuronosyltransferase (UGT) isoforms to buprenorphine and norbuprenorphine metabolism and relationship with the main UGT polymorphisms in a bank of human liver microsomes.

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Running title: glucuronidation of buprenorphine and norbuprenorphine

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Number of text pages: 32
Number of tables: 2
Number of figures: 3
Number of references: 37
Number of words in the abstract: 213
Number of words in the introduction: 491
Number of words in the discussion: 1404

Abbreviations: HLM, human liver microsomes; UGT, UDP-glucuronosyltransferase; BUP, buprenorphine; Nor-BUP, norbuprenorphine; AZT, 3'-azido-3'-deoxythymidine; ETO, etoposide; RAF, Relative Activity Factor; Cl_{max}, maximal clearance; V_{max}, maximal velocity; n, Hill coefficient; PCR, Polymerase Chain Reaction
Abstract

The goal of this study was to evaluate the specific contribution of individual UDP-glucuronosyltransferase (UGT) isoforms in the metabolism of buprenorphine (BUP) and norbuprenorphine (Nor-BUP), as well as the impact of their genetic variations. The glucuronidation of BUP and Nor-BUP was examined using human liver microsomes (HLM) and heterologously expressed UGTs. The individual contribution of UGT isoforms was estimated using enzyme kinetic experiments combined with the relative activity factor (RAF). Phenotype-genotype relationships were investigated in a bank of 52 human liver microsomes. Among the 6 hepatic UGT isoforms tested, UGT1A1, 1A3 and 2B7 metabolized BUP and Nor-BUP. Using the RAF approach, we found that UGT1A1 and UGT2B7 accounted for approximately 10% and 41% of BUP glucuronidation, respectively. Nor-BUP glucuronidation involved predominantly UGT1A3 (approximately 63%) and UGT1A1 (34%) whereas UGT2B7 had only a minor role. The UGT1A1 promoter (TA)₆/₇TAA mutation (UGT1A1*28) resulted in a 28% decrease of BUP glucuronidation $V_{\text{max}}$ in pooled HLM, but was not statistically associated with glucuronidation rate in 52 individual HLM. The presence of the UGT2B7 promoter (G-842A) mutation resulted in higher BUP glucuronidation $V_{\text{max}}$ in pooled HLM (+80% on average) and in a significant higher glucuronidation rate in non-carriers (but not in carriers) of the UGT1A1*28 allele (p=0.0352). This study represents a functional basis for further clinical pharmacogenetic studies.
INTRODUCTION

Buprenorphine (BUP) is a semi-synthetic derivative of the morphine alkaloid thebaine with partial agonist properties on opioid receptors. It is used at low doses (ranging from 0.3 to 0.6 mg) in the treatment of moderate to severe pain by intravenous, intramuscular and sublingual routes and at high–doses by the sublingual route (up to 16 mg day, in combination with naloxone in some countries) in opioid replacement therapy. In this indication, dose adjustment is critical since excessive doses can lead to adverse events (usually non-serious, but which may result in poor compliance) and alternatively inadequate doses usually result in treatment relapse. Although sublingual BUP has very low and variable bioavailability, and overall variable pharmacokinetics (Kuhlman et al., 1996), dose adjustment relies mainly on clinical monitoring. It is thought that genetic polymorphisms in drug metabolizing enzymes might contribute to the variability in BUP efficacy. Beside blood or urinary levels, patients genotyping may help improve *a priori* dose selection or *a posteriori* adjustment based on anticipated individual metabolic capacity.

The oxidative metabolism of BUP has been extensively studied. Rapid metabolism of BUP through N-dealkylation in the liver produces norbuprenorphine (Nor-BUP), an active metabolite (Huang et al., 2001). This pathway is mainly catalyzed by the cytochromes P450 (CYP) 3A4 and 2C8 (Iribarne et al., 1997; Kobayashi et al., 1998; Picard et al., 2005a). Subsequently, BUP and Nor-BUP undergo extensive phase II metabolism, catalyzed by UDP-glucuronosyltransferases (UGT) (Cone et al., 1984). This second pathway has received much less attention. King et al. (1996) demonstrated that BUP and Nor-BUP glucuronidation involves UGT1A1, although apparently at a different active site than that used for bilirubin (Rios and Tephly, 2002). In addition, it was shown that BUP and Nor-BUP are effectively glucuronidated by UGT2B7 and 1A3, respectively (Chang and Moody, 2009; Green et al., 1998; Rios and Tephly, 2002), Nor-BUP being a better substrate for UGT1A3 than BUP.
Inter-individual variability in BUP or Nor-BUP conjugation in the liver may influence BUP pharmacokinetics. Coffman et al. (1998) found, using HEK293 transfected cells, that the C to T substitution at nucleotide 802 (reference SNP ID number: rs7439366) of UGT2B7 giving rise to enzymes with either histidine (H) or tyrosine (Y) at the amino acid 268 position, alters the affinity for BUP. The consequence of this polymorphism on BUP hepatic glucuronidation remains to be investigated.

The insertion of a TA dinucleotide in the TATA-box of UGT1A1 promoter (referred as UGT1A1*28) is associated with the most common inherited cause of unconjugated hyperbilirubinemia, known as the Gilbert Syndrome. This polymorphism results in a 20-80% reduction in gene expression (Bosma et al., 1995; Monaghan et al., 1996). The role of this polymorphism in BUP glucuronidation has not been investigated so far.

The aim of the present study was to (i) evaluate qualitatively and quantitatively the specific contribution of hepatic UGTs in BUP and Nor-BUP glucuronidation and (ii) to assess the effect of their common genetic variants using a bank of human liver microsomes (n=52).
MATERIALS AND METHODS

Chemicals and Reagents

BUP, etoposide (ETO), mycophenolic acid (MPA), uridine 5'-diphosphoglucuronic acid (UDPGA), Triton X-100 and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St-Louis, MO, USA). Nor-BUP and BUP-glucuronide were purchased from Cerilliant (Molsheim, France). Mycophenolic acid acyl-glucuronide (AcMPAG) was a gift from Hoffman – La Roche Ltd. 3'-azido-3'-deoxythymidine (AZT, zidovudine) was a kind gift from GlaxoSmithKline (Nanterre, France). AZT-β-D-glucuronide was purchased from Toronto Research Chemical Inc. (Toronto, Canada). All solvents were HPLC-grade.

Enzymes and Microsomes

Microsomes prepared from baculovirus-infected insect cells expressing human UGT1A1, 1A3, 1A4, 1A6, 1A9, 1A10, 2B7 (Supersomes®), as well as a control preparation were obtained from BD Biosciences/GENTEST (Woburn, MA, USA).

In addition, 52 microsomal preparations were individually prepared from liver samples derived from surgical specimens obtained from Biopredic International (Rennes, France). All samples were collected after donors had given their informed consent, in accordance with the French bioethics law. Microsomes were obtained by differential centrifugation as previously described (Picard et al., 2004). Protein concentrations of the microsomal suspensions were measured according to the Bradford method using bovine serum albumin as standard. A pool derived from these 52 preparations was used in preliminary experiments.

Genotyping of Human Liver

Genomic DNA was extracted from each human liver sample used in microsome preparations as previously described (Picard et al., 2007) and genotyped for UGT2B7 G-842A single
nucleotide polymorphism (SNP) using a validated TaqMan allelic discrimination assay on an ABI PRISM 7000 Sequence Detection System (Applied-Biosystems, Courtaboeuf, France). Samples were also screened for TA insertion in the TATA-box of UGT1A1 promoter by direct sequencing. A 186-bp region containing the TATAA element was amplified by PCR using 50 ng of DNA, AmpliTaq Gold Master Mix (Applied-Biosystems) and the following primers: 5’-TCCCTGCTACCTTTGTGGAC-3’ (forward) and 5’-AGCAGGCCAAGGACAAGT-3’ (reverse). Amplification consisted of an initial denaturation for 8 minutes at 93°C followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 1 minute. Terminal elongation was performed at 72°C for 7 minutes. The sequencing reaction was carried out using the BigDye Terminator 1.1 Cycle Sequencing Kit (Applied-Biosystems) using 4 µl of the purified primary PCR product, 4 µl of BigDye Terminator and 2 µl of reverse primer (2.5 µM). Reactions included denaturation at 93°C for 8 minutes, followed by 35 cycles at 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 1 minute. Extension products were purified using a standard Ethanol/EDTA/sodium acetate precipitation procedure and the nucleotide sequences determined on an ABI 3100 automated sequencer (Applied-Biosystems).

Assay with Microsomes and Recombinant UGT Enzymes

The typical incubation mixture (250 µl) contained 0.2 mg/ml microsomal proteins (HLM or Supersomes®), 1 mM UDPGA, 10 mM MgCl₂, 5 µM BUP or Nor-BUP, and 0.1 M TRIS-HCl buffer (pH 7.4). Microsomes were first detergent-activated by incubation with Triton X-100 during 30 minutes on ice, at an optimized detergent-to-microsomal protein ratio of 0.4 (w/w). BUP and Nor-BUP were then pre-incubated with microsomes at 37°C for 5 minutes and the reaction initiated by addition of UDPGA. After 20 minutes incubation at 37°C, the reaction
was stopped by addition of 100 µl ice-cold acetonitrile. Samples were centrifuged and stored at –20°C until analysis. Control incubations without microsomes or without UDPGA were performed in parallel to each experiment.

**Enzyme Kinetic Experiments**

In preliminary experiments with pooled HLM, the linearity of metabolites formation with increasing microsomal protein concentration (0.1-1 mg/ml), and incubation time (10-60 minutes) was checked. Kinetic experiments were then performed by incubating increasing concentration of BUP (0.5-75 µM) for 20 minutes with 0.2 mg/ml microsomes (total pool and pools of specific genotype). Kinetic data were model-fitted using the Hill equation and kinetic parameters calculated by non-linear regression analysis using Winreg 3.1 (available online: [http://www.unilim.fr/pages_perso/jean.debord/winreg/winreg1.htm](http://www.unilim.fr/pages_perso/jean.debord/winreg/winreg1.htm)).

Maximal clearances (Cl\textsubscript{max}) were estimated using Equation 1 (Houston and Kenworthy, 2000) where \( V_{\text{max}} \) is the maximal velocity rate, \( S_{50} \) the substrate concentration resulting in 50% of \( V_{\text{max}} \) and \( n \) is the Hill coefficient:

\[
\frac{V_{\text{max}}}{S_{50}} \times \frac{(n - 1)}{n(n - 1)^{1/n}}
\]

Since Nor-BUP was only available as a 100 µg/ml solution in methanol (Cerilliant, France), we could not prepare solutions at high concentrations and kinetic experiments could not be performed with this compound.

**LC-MS/MS analysis**

BUP and Nor-BUP metabolites were determined using LC-MS/MS. Sample preparation consisted of adding 10 µl internal standard (glafenin 2 mg/l) to 90 µl of incubation supernatants. Samples (10 µl) were then injected into the chromatographic system that consisted in a Perkin-Elmer Series 200 autosampler equipped with a Rheodyne Model 7725.
DMD #29546

injection valve with a 5-µl internal loop, an Agilent HP1100 high-pressure gradient pumping system and a Waters Atlantis T3, 5 µm (150 x 2.1 mm) column maintained at 25°C. A linear gradient of mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in methanol) was delivered at a constant flow rate of 200 µl/min and programmed as follows: 0-1.5 min, 3% B; 1.5-2.5 min, 3% to 55% B; 2.5-3.5 min, 55% to 60% B; 3.5-4.5 min, 60% to 90% B; 4.5-6.5 min, 90% B; 6.5-7 min, decrease from 90% to 3%; 7-9 min, 3% B.

Detection was performed using an Applied Biosystems 4000 QTRAP™ LC-MS/MS System, equipped with a Turbo V ionization source and controlled by Analyst 1.5 software. Positive ionization was performed with the following settings: ion spray voltage, 5500 V; curtain gas, 15; ion source gas flow rates 1 and 2 at 20 and 30 units, respectively; and temperature set at 500°C. Acquisitions were made in the multiple reaction monitoring (MRM) mode using two transitions for BUP (m/z 468→55; m/z 468→83) and Nor-BUP (m/z 414→55; m/z 414→83), three for their respective glucuronides (BUP-glucuronide: m/z 644→468; m/z 644→55; m/z 644→83, Nor-BUP glucuronide: m/z 590→414; m/z 590→55; m/z 590→83) and two for the internal standard, glafenin (m/z 373→218; m/z 373→281). The limit of quantification was 5 µg/l for BUP, Nor-BUP and BUP-glucuronide. Calibration curves obtained using quadratic regression from the limit of quantitation up to 5000 µg/l yielded r > 0.99. In the absence of pure Nor-BUP-glucuronide, the concentration of this metabolite was estimated as molar equivalent with respect to Nor-BUP calibration curves.

**UGT1A1 and UGT2B7 RAF Determination**

The Relative Activity Factor (RAF) approach is proposed for scaling enzymatic activities obtained using cDNA-expressed enzymes to HLM. RAFs are defined as the HLM/recombinant enzyme activity ratio of a particular isoform towards a probe substrate
following experiments performed under identical conditions. The relative amount of specific substrate metabolism attributed to individual enzymes (contribution) is estimated by multiplying the rate of metabolism observed with this enzyme ($V_i$) by the corresponding RAF (Equation 2).

$$\text{Contribution}_i = V_i \times \text{RAF}_i$$

(2)

The RAF approach is well established for cytochrome P450-dependant activity (Crespi and Miller, 1999). In this study, we calculated RAFs for UGT1A1 and UGT2B7 in order to scale velocities obtained using UGT Supersomes® to HLM. Based on incubation with a panel of UGT Supersomes®, we first confirmed that, in our incubation conditions, ETO and AZT were selective substrates for UGT1A1 and UGT2B7, respectively (data not shown). MPA was used as a second probe substrate for UGT2B7 since this isoform catalyzes the production of AcMPAG in a specific manner (Picard et al., 2005b).

In the case of UGT1A3, the RAF could not be calculated as no adequate probe was identified. Incubations were performed as follows: HLM (0.5 mg/ml) and recombinant UGT (0.25 mg/ml) were activated by preincubation with Triton X100 as described above. Microsomes were then incubated with ETO (25 µM) and AZT (25 µM) at 37°C in 0.1 M Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂, and 2 mM UDPGA, in a total volume of 125 µl. Incubations were carried out for 60 minutes and terminated by addition of 100 µl of ice-cold methanol containing glafenin (2.7 µM) as an internal standard. Samples were centrifuged at 10000 g for 5 minutes at 4°C to pellet the precipitated protein. ETO and AZT glucuronides were determined using LC-MS/MS. The chromatographic and detection systems were similar to those described for BUP and Nor-BUP metabolite determinations. A linear gradient of mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in methanol) was delivered at a constant flow rate of 200 µl/min and programmed as follows: 0-1 min, 3% B; 1-2 min, 3% to 10% B; 2-4 min, 20% to 40% B; 4-5 min, 40% to
70% B; 5-10 min, 70% to 90% B; 10-12 min, 90% B; 12-13 min, decrease from 90% to 3%; 13-17 min, 3% B. The electrospray ionization source was operated in positive mode with the following settings: ion spray voltage, 5500 V; curtain gas, 20; ion source gas flow rates 1 and 2 at 15 and 35 units, respectively and temperature set at 500°C. MS/MS detection was carried out in the MRM mode, following two transitions for ETO (m/z 590 → 229; m/z 606 → 229) and AZT (m/z 268 → 110; m/z 268 → 127), three for the glucuronides of ETO (m/z 766 → 185; m/z 782 → 185; m/z 782 → 229) and of AZT (m/z 444 → 110; m/z 444 → 127; m/z 444 → 268) and two for the internal standard, glafenin (m/z 373 → 218; m/z 373 → 281). For AZT-glucuronide quantification, a standard curve from 5 to 500 µg/L was constructed. Because ETO-glucuronide was not commercially available, its concentration was estimated as molar equivalent with respect to ETO calibration curves. Quantification was carried out using a weighted (1/x) quadratic regression model, yielding correlation coefficients >0.99.

Incubations of MPA with HLM (0.5 mg/ml) or recombinant UGT (0.25 mg/ml), and AcMPAG LC-MS/MS determination were performed as described previously (Picard et al., 2005b).

RAF values were calculated by dividing the mean activity of glucuronide formation (n=3 experiments) obtained in HLM by that obtained in Supersomes® (both expressed in pmol/mg protein/min). Contributions of UGT1A1 and 2B7 were calculated using eq.2.

**Statistical Analysis**

The distributions of UGT1A1 and UGT2B7 alleles were compared to the Hardy–Weinberg theoretical distribution using the Fisher’s exact test. All activities were measured at least in duplicate and averaged. For both genes, because of the limited number of samples, data obtained with microsomes of the homozygous variant and heterozygous genotypes were grouped for statistical analyses. Microsomal velocity rates between genotype groups were
then compared using ANOVA in the StatView program (SAS Institute, Cary, NC, version 5.0) with a level of significance set at 0.05. The effect of the \textit{UGT2B7} polymorphism was also investigated after stratification of the data by the \textit{UGT1A1} genotype (i.e. carriers and non-carriers of the \textit{UGT1A1*28} allele). In this case, the non-parametric Mann-Whitney test was used with $p<0.05$ considered as statistically significant.
RESULTS

UGT Isoforms Involved in BUP and Nor-BUP Glucuronidation

As shown in Figure 1, incubation of BUP or Nor-BUP with recombinant UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A9 and 2B7) revealed that only UGT1A1, 1A3 and 2B7 were able to produce BUP and Nor-BUP glucuronides in detectable amounts. However, their respective activities regarding BUP and Nor-BUP were different. Recombinant UGT1A1 metabolized BUP with the highest efficiency whereas UGT1A3 was the most efficient for Nor-BUP. The activity of recombinant UGT2B7 towards Nor-BUP was very low in comparison to that of UGT1A1 and 1A3 (Figure 1). In contrast, UGT2B7 showed the second highest activity in BUP metabolism.

Kinetics of BUP Glucuronide Production by UGT1A1, 1A3 and 2B7

The kinetic constants of BUP glucuronidation were calculated using the three competent recombinant enzymes. Sigmoidal kinetics was observed with the three UGTs, as well as with HLM. As shown in Table 1, the affinity of UGT2B7 was 2- and 3.4-fold higher than that of UGT1A3 and UGT1A1, respectively. In contrast, the maximal velocity (V_max) of BUP-glucuronide production was much higher for UGT1A1 than for UGT1A3 and UGT2B7 (Table 1).

The velocity rates obtained with recombinant UGT1A1 and UGT2B7 were scaled to HLM using the RAF calculated from the UGT1A1-mediated glucuronidation of ETO (RAF=0.6) and the averaged RAF (=6.8) obtained from the UGT2B7-mediated glucuronidation of MPA (RAF=4.5) and AZT (RAF=9.0). As can be seen in Table 1, the scaled Cl_max of UGT2B7 was 4.1-fold higher than that of UGT1A1, each representing 41% and 10% of the Cl_max of HLM,
respectively. This left approximately 49% of BUP-glucuronide production in HLM not attributable to UGT1A1 and UGT2B7.

Standard kinetic experiments could not be performed for Nor-BUP because no powder formulation of Nor-BUP was commercially available, preventing us from preparing solutions at high enough concentrations. As a surrogate approach, the velocity of Nor-BUP-glucuronidation obtained with recombinant enzymes and HLM at a low single concentration of substrate (5 µM) were compared. The scaled velocity of Nor-BUP glucuronidation obtained with UGT1A1 (17.4 ± 1.2 pmol/mg protein/min) represented 34% of the velocity obtained in HLM (51.8 ± 1.8 pmol/mg protein/min). UGT2B7 activity was barely detectable (i.e., the metabolic rate was lower than the limit of quantitation of the assay). The activity observed for UGT1A3 (32.5 ± 0.8 pmol/mg/min) could not be scaled to HLM directly, in the absence of a RAF for this isoform. However, it was inferred by difference between the measured HLM activity and the UGT1A1 and UGT2B7 activities, representing approximately 65%.

Influence of UGT1A1*28 and UGT2B7 G-842A genotypes on BUP glucuronidation in HLM

Genotype distribution among the banked microsomes

UGT1A1 and UGT2B7 genotype distributions were in Hardy-Weinberg equilibrium and similar to those reported previously (Djebli et al., 2007; Iyer et al., 1999). UGT1A1*28 allelic frequency was 0.37, with 21 microsomal preparations (40%) carrying the TA₆/TA₆ (wild type) genotype, 24 (46%) the heterozygous genotype and 7 (13%) the TA₇/TA₇ (variant) genotype. UGT2B7 G-842A polymorphism frequency was 0.53, with 11 (21%), 26 (50%) and 15 (29%) preparations of the wild-type (GG-842), heterozygous and variant genotype, respectively.
BUP glucuronidation by genotyped microsomes

BUP was incubated with each of the 52 microsomal preparations at a single concentration of 5 µM. Velocities varied up to 3.4-fold (mean±SD: 2.1±0.6-fold). Using two-way analysis of variance (ANOVA), a trend was found for UGT2B7 G-842A (p=0.0722), while no significant difference in BUP-glucuronidation was found for the UGT1A1 polymorphism (p=0.3214). However, as there was an interaction between the two SNPs (p=0.0199), we investigated the effect of the UGT2B7 polymorphism within each UGT1A1 genotypic group, showing a significant effect of UGT2B7 on BUP-glucuronidation in the UGT1A1 homozygous wild-type group (p=0.0352) and none in UGT1A1*28 allele carriers (p=0.4532) (Figure 2).

Kinetics of BUP glucuronidation by pools of genotyped HLM

Three microsome pools were constituted using the following UGT2B7 genotype: GG-842 (n=5), G-842A (n=10) and -842AA (n=6). The microsomes used in these 3 pools were selected to be non-carrier of the UGT1A1*28 allele. Another pool was prepared from HLM homozygous for the UGT1A1*28 allele (n=7), comprising 1, 4 and 2 preparations of the UGT2B7 wild-type, heterozygous and variant genotype, respectively. The distribution of the UGT2B7 variant allele in the UGT1A1*1 wild-type and mutated groups were thus similar (allelic frequency=0.52 vs 0.57; Fisher-Exact p >0.999).

BUP glucuronidation kinetic parameters were estimated in each pool (Table 2 and Figure 3). All the preparations displayed comparable S_{50} values, similar to that obtained with the pool of 52 HLM. The V_{max} obtained with the pool of mutated microsomes for UGT1A1 was 1.4-fold lower than the average V_{max} in wild-type preparations. This resulted in a 1.3-fold lower Cl_{max}. The effect of the UGT2B7 G-842A polymorphism was assessed with a pool of microsomes wild-type for UGT1A1. The pool of heterozygous microsomes for UGT2B7 G-842A showed
approximately the same $V_{\text{max}}$ as the homozygous mutated one, both being approximately 1.7-fold higher than that of the homozygous wild-type microsomes.
DISCUSSION

We performed experiments with human recombinant enzymes and confirmed that, among the six hepatic UGT tested, only UGT1A1, 2B7 and 1A3 were involved in BUP and Nor-BUP metabolism. Kinetic experiments showed that their respective contribution was, in decreasing order: UGT 1A3>2B7>1A1 for BUP; UGTs 1A3>1A1>2B7 for Nor-BUP. The UGT1A1 promoter polymorphism (TA)$_{6/7}$TAA (UGT1A1*28) had a moderate effect on BUP glucuronidation $V_{\text{max}}$ in pooled HLM, but it was not statistically associated with BUP glucuronidation in 52 individual HLM. The presence of the UGT2B7 promoter (G-842A) mutant allele resulted in higher $V_{\text{max}}$ in pooled HLM and significantly higher BUP glucuronidation in individual microsomes non-carriers (but not in carriers) of the UGT1A1*28 allele.

Although UGT1A1, 1A3 and 2B7 were already highlighted as competent isoforms for BUP (Cheng et al., 1998; Coffman et al., 1997; Coffman et al., 1998; Green et al., 1998; King et al., 1996; King et al., 1997; Rios and Tephly, 2002; Soars et al., 2003) and Nor-BUP glucuronidation (King et al., 1997), their specific contributions were unclear. Simultaneously investigating the metabolism of BUP and its primary phase I metabolite Nor-BUP allowed us to compare the UGTs activity toward the two compounds. The same isoforms metabolized BUP and Nor-BUP, but with different relative activities, consistent with results recently published by Chang and Moody showing that UGT1A1 and 1A3 are preferentially involved in Nor-BUP metabolism (Chang and Moody, 2009). We found here that UGT1A3 and 2B7 are predominantly involved for BUP, whereas UGT1A1 only plays a minor role. Concerning UGT1A3 more specifically, we found, as already reported by Green et al. (1998), that it had a higher activity for Nor-BUP than for BUP.

The major limitation of recombinant enzymes or transfected cells in drug metabolism studies is that extrapolation of the results to humans requires taking into account specific factors such...
as differences in membrane compositions between expression models and hepatocytes, the absence of competing enzymes and above all the relative expression level of enzyme isoforms in the liver. To overcome this limitation, an extrapolation method has been proposed for P450 enzymes (Crespi and Miller, 1999; Venkatakrisnan et al., 2000), consisting in relative activity factors (namely “RAF”) allowing extrapolation of recombinant enzyme formation rates to native human liver enzyme activity. Toide et al. previously proposed a RAF determination method for UGT enzymes (Toide et al., 2004). Since UGTs exhibit overlapping substrate affinities and probe substrates had not been identified for each UGT, the authors calculated RAF using the activity of different recombinant UGTs towards selective and non-selective substrates using a system of equations.

In this study, we used the RAF approach proposed for P450 by Crespi et al. in order to quantitatively evaluate the role of UGT1A1 and 2B7 in BUP and Nor-BUP metabolism. RAfs were calculated from the metabolic rates obtained by incubating ETO, AZT and MPA with recombinant enzymes and with a large pool of HLM preparations. The choice of these substrates was supported by the literature (Barbier et al., 2000; Picard et al., 2005b; Watanabe et al., 2003) and experiments performed herein showing that ETO and AZT were specific of UGT1A1 and 2B7, respectively. RAF values obtained from UGT2B7-mediated glucuronidation of AZT (RAF=9.0) and MPA (RAF=4.5) showed that the activity of heterologously-expressed UGT2B7 was lower than that of native hepatic UGT2B7. Conversely, the RAF value obtained from UGT1A1-mediated glucuronidation of ETO (RAF=0.6) was less than unity, demonstrating that recombinant UGT1A1 has a much higher activity than native hepatic UGT1A1, which is in accordance with the RAF value of 0.31 found by Toide et al. (2004) using UGT1A1 Supersomes® and bilirubin as a probe substrate.
Using the RAF approach, we found that UGT1A1 accounted for approximately 10% and 30% of BUP and Nor-BUP glucuronidation, respectively, while UGT2B7 would be responsible for at least 40% of BUP-glucuronidation, with a very limited role in Nor-BUP glucuronidation. Because of the absence of RAF for UGT1A3, we can only hypothesize that this isoform would account for the remaining part of the metabolism of BUP (approximately 50%) and Nor-BUP (approximately 65%) in HLM.

We cannot exclude that other isoforms can contribute to BUP metabolism. Chang and Moody recently showed that UGT2B17 is a competent isoform in BUP metabolism (Chang and Moody, 2009). This isoform glucuronidates a large number of xenobiotics (Beaulieu et al., 1996; Lazarus et al., 2005; Turgeon et al., 2003) and its expression was found in various tissues including liver, kidney, placenta, uterus, small intestine and colon (Beaulieu et al., 1996; Ohno and Nakajin, 2009). However, the expression level of UGT2B17 in the liver is approximately 5% that of UGT2B7 (Ohno and Nakajin, 2009), suggesting a minor role in BUP hepatic metabolism.

Altogether, our results allow us to narrow-down the investigation of candidate genetic polymorphisms with potential consequences on BUP metabolism. The UGT2B7 promoter G-842A SNP is a frequent polymorphism, described as functional. It is strongly linked to other promoter variants including the -1248G, -268G, and -102C SNPs, and is inversely linked to the C802T exonic SNP (Djebli et al., 2007; Duguay et al., 2004) known to alter UGT2B7 affinity for BUP (Coffman et al., 1998). As described in the introduction section, the UGT1A1*28 promoter polymorphism was also described to contribute to variability in UGT1A1 expression level and activity (Beutler et al., 1998; Lampe et al., 1999; Peterkin et al., 2007). However, its effect on BUP metabolism has not been reported to date. Kinetic experiments performed with pools of genotyped HLM revealed that the presence of at least one mutated allele in UGT2B7 increased the $V_{\text{max}}$ 1.7-fold as compared to homozygous
wild-type microsomes suggesting that the level of gene expression is modified by the G-842A regulatory SNP. These results are identical to those of Djebli et al. (2007) for mycophenolic acid acyl-glucuronidation. The effect of UGT2B7*2 (C802T) was already studied on opioids and other substrates using HEK293 transfected cells: Coffman et al. demonstrated that the affinity of the variant protein UGT2B7 268H for BUP and morphine was altered (Coffman et al., 1998). The present results suggest that the effect of the promoter SNP (in complete inverse linkage with UGT2B7*2) is dominant, as V_{max} was increased while affinity was not altered. Such an effect on enzyme expression could not be detected using HEK293 transfected cells.

As for UGT1A1, the pool of microsomes carrying the UGT1A1*28 allele showed a modest decrease in V_{max}, in agreement with most previous studies on bilirubin or SN-38 (the active metabolite of irinotecan) glucuronidation (Bosma et al., 1995; Iyer et al., 1999; Monaghan et al., 1996) and consistent with the modest implication of UGT1A1 in BUP glucuronidation found here (10% based on the RAF approach).

In order to assess whether these changes in UGT1A1 and UGT2B7 velocity would translate into different BUP liver glucuronidation rates, BUP was incubated with 52 individual genotyped HLM. The UGT1A1*28 allele was not associated with a significant difference in BUP glucuronidation, suggesting that the Gilbert Syndrome would only have a limited effect on BUP liver glucuronidation. It was previously shown that BUP glucuronidation was drastically decreased in microsomes prepared from Crigler-Najjar type I patients’ liver samples (n=2) as compared to other patients’ liver microsomes (n=3) (King et al., 1996). However, the Crigler-Najjar Syndrome that also concerns UGT1A1 is more severe (but much rarer) than the Gilbert Syndrome as it results in a near (type I) or complete (type II) loss of enzyme activity.
In these 52 microsomes, the \textit{UGT2B7} G-842A polymorphism was associated with a trend to higher glucuronidation rates of BUP, an effect significant in non-carriers but not in carriers of the \textit{UGT1A1} *28. We have no clear explanation for this apparent difference in UGT2B7 effect depending on the \textit{UGT1A1} status. It may only indicate that the effect of the \textit{UGT2B7} G-842A SNP is relatively modest with respect to the overall variability in UGT activity.

To sum up, we have qualitatively and quantitatively evaluated the contribution of UGT hepatic isoforms in the metabolism of BUP and Nor-BUP and investigated the effect of their polymorphisms. We suggest that polymorphisms in \textit{UGT1A1} and 2B7 might not notably influence the disposition of BUP in humans. Since phase I metabolism is known to modulate the extent of phase II metabolism, it would be interesting to evaluate the simultaneous effect of P450 and UGT polymorphisms in BUP metabolism. For that, an integrated model such as genotyped HLM, and experimental conditions involving simultaneous incubation with phase I and phase II co-substrates such as that recently described by Kilford et al. (2009), might be particularly suited.
Acknowledgments

We would like to thank Biopredic International for their support.

We are extremely grateful to Jean-Hervé Comte for his technical assistance.
REFERENCES


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Footnotes

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FIGURE LEGENDS

**Figure 1:** BUP and Nor-BUP glucuronidation with different recombinant human hepatic UGTs. The final concentration of BUP and Nor-BUP in incubations was 5 µM. Data (means of individual duplicates) are expressed as activities relative to the most active isoform (i.e. UGT1A1 for BUP and 1A3 for Nor-BUP).

**Figure 2:** Box and whisker plot of the impact of *UGT1A1*<sup>*</sup>28 and *UGT2B7* G-842A genotypes on mean BUP-glucuronide metabolic rate by 52 genotyped human liver microsomes (middle lines represent the median, the upper and lower extremities of the box represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles).

**Figure 3:** Enzyme kinetic modeling of BUP glucuronidation using the Hill function in different pools of HLM genotyped for *UGT1A1*<sup>*</sup>28 and *UGT2B7* G-842A. Velocity rates are presented as the mean of duplicates.
Table 1: Glucuronidation kinetic parameters (mean estimate ± standard error) computed from incubation of BUP with HLM and competent human recombinant UGT isoforms.

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$</th>
<th>$S_{50}^a$</th>
<th>$n^a$</th>
<th>$Cl_{max}^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg protein</td>
<td>µM</td>
<td></td>
<td>µl/min/mg protein</td>
</tr>
<tr>
<td>HLM</td>
<td>1644.0±81.0</td>
<td>11.0±1.4</td>
<td>2.2±0.5</td>
<td>75.2</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>1768.9±240.0</td>
<td>71.8±12.4</td>
<td>1.7±0.17</td>
<td>7.5$^c$</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>313.3±34.3</td>
<td>43.1±6.8</td>
<td>1.8±0.3</td>
<td>-</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>186.5±11.9</td>
<td>20.9±2.5</td>
<td>2.5±0.6</td>
<td>30.7$^c$</td>
</tr>
</tbody>
</table>

$^a$ $S_{50}$ and $n$ (Hill coefficient) computed by Hill equation

$^b$ $Cl_{max}$ calculated as described under Material and Methods.

$^c$ Scaled $Cl_{max}$ to HLM using RAF approach
Table 2: Kinetic parameters (mean estimate ± standard error) of BUP-glucuronidation in pools of HLM genotyped for *UGT1A1*28 and *UGT2B7* G-842A.

<table>
<thead>
<tr>
<th>Pool of genotyped HLM</th>
<th>$V_{\text{max}}$</th>
<th>$S_{50}^{a}$</th>
<th>$Cl_{\text{max}}^{b}$</th>
<th>$n^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein/min</td>
<td>µM</td>
<td>µl/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>GG-842 (n=5)</td>
<td>1689.6 ± 39.9</td>
<td>11.2 ± 0.6</td>
<td>75.2</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>G-842A (n=10)</td>
<td>2725.0 ± 131.1</td>
<td>10.6 ± 1.1</td>
<td>134.4</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>-842AA (n=6)</td>
<td>2817.0 ± 131.2</td>
<td>10.6 ± 1.1</td>
<td>136.2</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Mixed results$^{c}$</td>
<td>2400.3 ± 89.4</td>
<td>10.7 ± 0.8</td>
<td>115.2</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>UGT1A1*28/*28 (n=7)</td>
<td>1726.7 ± 69.6</td>
<td>9.5 ± 0.8</td>
<td>91.4</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

$^{a}$ $S_{50}$ and $n$ (Hill coefficient) computed by Hill equation.

$^{b}$ $Cl_{\text{max}}$ calculated as described under Material and Methods.

$^{c}$ Computed from the average of the velocities observed in the UGT2B7 genotyped pools.
Figure 1
Figure 2

Boxplots showing the BUP-Glucuronide metabolic rate for different conditions. The p-value is 0.0352.

- 1A1 (wt) / 2B7 (mut) (n=16)
- 1A1 (wt) / 2B7 (wt) (n=5)
- 1A1 (mut) / 2B7 (wt) (n=6)
- 1A1 (mut) / 2B7 (mut) (n=25)
Figure 3