Published in final edited form as:

Pharmacogenet Genomics. 2012 March ; 22(3): 183–190. doi:10.1097/FPC.0b013e32834ff3a5.

Glucuronidation of trans-3'-hydroxycotinine by UGT2B17 and UGT2B10

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Abstract

trans-3'-Hydroxycotinine (3HC) and its glucuronide (3HC-Gluc) are major nicotine metabolites excreted in the urine of smokers and other tobacco users. While several members of the UDP-glucuronosyltransferase (UGT) family of enzymes were previously shown to be active in catalyzing the formation of 3HC-Gluc, a comprehensive screening of all known human UGT1A and 2B enzymes for glucuronidation activity against 3HC was not previously performed. In the present study, 8 UGT1A and 6 UGT2B enzymes were screened for activity against 3HC. UGT2B17 exhibited the highest *O*-glucuronidation activity, exhibiting a 4-fold lower ($p < 0.005$) K_M (8.3 mM) than that observed for UGTs 1A9 (35 mM) or 2B7 (31 mM) and a K_M smaller than that observed for human liver microsomes (HLM; 26 mM). The K_M for 3HC-*O*-Gluc formation was 3.1-fold lower ($p < 0.0005$) in HLM from male subjects exhibiting the wild-type genotype *UGT2B17* (*1/*1) than that in HLM from subjects homozygous for the *UGT2B17* deletion genotype [*UGT2B17* (*2/*2)]. Both UGTs 2B10 and 1A4 exhibited 3HC-*N*-Gluc formation activity, with UGT2B10 exhibiting a 4-fold lower ($p < 0.05$) K_M (13 mM) than that observed for UGT1A4 (57 mM) and which was similar to the K_M observed in HLM (14 mM). There was a 91% ($p < 0.0001$) and 39% ($p < 0.001$) decrease in 3HC-*N*-Gluc formation activities in HLM from subjects with the *UGT2B10* (*2/*2) and *UGT2B10* (*1/*2) genotypes, respectively, compared to that of HLM from subjects with the wild-type *UGT2B10* (*1/*1) genotype. These results suggest that UGT2B17 and UGT2B10 play key roles in the glucuronidation of 3HC in the human liver and that functional polymorphisms in *UGT2B17* and *UGT2B10* are associated with significantly reduced glucuronidation activities against 3HC.

Introduction

Tobacco smoking causes 5 million deaths annually worldwide, and nicotine is the single most important pharmacological agent responsible for tobacco addiction (1). The prevention and treatment of tobacco addiction requires a greater understanding of the pharmacologic properties of nicotine including its metabolic pathways. *In vivo*, nicotine is primarily metabolized to cotinine, which is further metabolized to trans-3'-hydroxycotinine (3HC) and other compounds [see Figure 1; (2, 3)]. Nicotine, cotinine and 3HC undergo further phase II detoxification reactions by conjugation with glucuronic acid via catalysis by the UDP-glucuronosyltransferase (UGT) family of enzymes (3). Glucuronidation occurs on the nitrogen of the pyridine ring for both cotinine and nicotine (4–7) and UGT2B10 was shown to be the most active enzyme in this catalysis (8). UGT2B10 was further implicated in nicotine and cotinine metabolism due to a functional polymorphism (Asp69Tyr) being

highly associated with decreased activity against both substrates in human liver microsomes (HLM), and with decreased levels of both nicotine and cotinine in the urine of smokers (2). While UGT1A4 was shown to be active in 3HC-*N*-glucuronide (3HC-*N*-Gluc) formation (9, 10), UGT2B10's role in 3HC glucuronidation was not previously studied.

Like nicotine and cotinine, 3HC can be metabolized on the nitrogen of the pyridine ring, but it is also *O*-glucuronidated on its 3'-hydroxyl group to form 3HC-*O*-glucuronide (3HC-*O*-Gluc). Similar to that observed in previous studies for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the major metabolite of the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), both UGTs 2B7 and 1A9 were active in 3HC-*O*-Gluc formation (11). However, UGT2B17, which exhibited the highest level of activity of all hepatic UGTs activity against NNAL, was not previously screened for activity against 3HC. The polymorphic whole-gene deletion of the *UGT2B17* gene was associated with reduced *O*-glucuronidation activity against NNAL in HLM (12). As 3HC is structurally-related to NNAL and because UGT2B17 is expressed in liver (13, 14), we hypothesized that UGT2B17 may play an important role in hepatic 3HC glucuronidation.

UGT2B10 is highly expressed in human liver (15). Recent studies indicated that UGT2B10 was high active against nicotine, cotinine and tobacco-specific nitrosamines to form *N*-glucuronides, and a functional polymorphism of *UGT2B10* (*Asp67Tyr*) was associated with reduced *N*-glucuronidation activities against NNAL, nicotine and cotinine in HLM (16). The *UGT2B10* codon 67 polymorphism was also shown to be associated with reduced levels of nicotine-*N*-Gluc and cotinine-*N*-Gluc in smokers' urine (2). As 3HC structurally similar to nicotine and cotinine, we hypothesized that UGT2B10 may play an important role in 3HC *N*-glucuronidation in HLM. The goal of the present study was to perform a comprehensive analysis of known human UGTs including UGTs 2B17 and 2B10 against 3HC, and study the potential effects of functional polymorphisms in UGTs 2B17 and 2B10 genes on 3HC glucuronidation activities.

Materials and Methods

Chemicals

3HC, 3HC-*O*-Gluc, 3HC-*N*-Gluc and the deuterium-labeled internal standards cotinine-(methyl-D₃)-3'-*O*-glucuronide were purchased from Toronto Research Chemicals Inc (North York, Canada). UDPGA, alamethicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bicinchoninic acid (BCA) assay was purchased from Pierce (Rockford, IL, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Tissues and cell lines

Normal human liver specimens and matching genomic DNA samples were obtained from 110 subjects and liver microsomes were prepared using differential centrifugation and stored (10–20 mg protein/mL) at –80 °C as previously described (17). All 110 subjects from whom liver specimens were obtained were Caucasian, and 43% (n=47) were female. Microsomal protein concentrations were measured using the BCA assay. HEK293 cells individually over-expressing UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15 and 2B17 have also been previously described (18–20). Cell homogenates were prepared by re-suspending pelleted cells in Tris-buffered saline [25 mM Tris-HCl (pH 7.4), 138 mM NaCl, 2.7 mM KCl] and subjecting them to 3 rounds of freeze-thaw prior to gentle homogenization. All cell homogenates, HLM and DNA samples were stored at –80°C.

Glucuronidation assays and analysis

Glucuronidation activities of HLM or homogenates from UGT-over-expressing cell lines were determined after an initial incubation of HLM (5–25 µg protein) or UGT-over-expressing cell homogenates (50 µg protein) with alamethicin (50 µg/mg protein) for 15 min in an ice bath. Briefly, 3HC at different concentrations were incubated with HLM or cell homogenates in 50 mM Tris buffer (pH 7.5), 10 mM MgCl₂ and 4 mM UDPGA. Incubations (10 µL) were performed at 37°C for 1 h, a time that was found in previous studies to be within the linear range of product formation for the UGTs tested in this study. After incubations, reactions were first spiked with 5 µL of internal standard cotinine-(methyl-D₃)-3'-O-glucuronide (10 ppm) and were terminated by the addition of 60 µL of cold acetonitrile/methanol (75%/25% [v/v]). After vortexing and subsequent centrifugation at 12,000 g for 10 min at 4°C, 50 µL of supernatant was transferred to a sample vial for analysis by ultra-pressure liquid chromatography (UPLC)-mass spectrometry (MS) analysis using an Acquity LC-MS/MS system (Waters Corporation, Milford, MA, USA) consisting of an Acquity UPLC pump, an auto sampler, an ACQUITY UPLC BEH HILIC column (2.1 mm × 100 mm, 1.7 µm particle size; Waters Corp.), and an Acquity TQ tandem mass spectrometer (Waters). UPLC was performed at a flow rate of 0.5 mL/min using the following conditions: 1.5 min in 20% solvent A, a linear gradient for 2 min to 100% solvent A, and 2 min in 100% solvent A, where solvent A is 5 mM NH₄AC (pH 5.7)/50% acetonitrile (v/v) and solvent B is 5 mM NH₄AC (pH 5.7)/90% acetonitrile (v/v). After each run the column was reconditioned with 20% solvent A for 1 min at a flow rate of 1 mL/min. The column was maintained at 45°C, and the injection volume of each prepared sample was 5 µL using a partial loop injection mode.

The operation conditions for the Waters Acquity TQ tandem MS was as follows: the electrospray ionization (ESI) probe was operated in the positive-ion mode, with capillary voltage at 0.64 kV. Nitrogen was used as both the cone and desolvation gases with flow rates maintained at 20 and 760 L/h, respectively. Ultra-pure argon was used as the collision gas with a flow rate of 0.1 L/h for collision-induced dissociation. The source and desolvation gas temperatures were 140 °C and 450°C, respectively. For determination of the concentration of 3HC-*O*-Gluc and 3HC-*N*-Gluc in 3HC glucuronidation assay samples, the mass spectrometer was operated in the multiple reaction monitoring mode (MRM). The dwell time for each ion was 100 ms with 5 ms of inter-scan-delay. The cone voltage and collision energy were 20 and 15 V, respectively, for both 369 > 193 (3HC-*O*-Gluc or 3HC-*N*-Gluc) and 372 > 196 (internal standard) ion transitions.

For quantification of peaks, standard curves were constructed by plotting the ratio of 3HC-*N*-Gluc or 3NC-*O*-Gluc peak area to the peak area of deuterium-labeled internal standard, versus analyte concentrations ranging from 0.2 ppm to 25 ppm. Actual 3HC-*O*-Gluc and 3HC-*N*-Gluc concentrations were determined by measuring the 3HC-*N*-Gluc or 3HC-*O*-Gluc peak area ratios to the internal standard [cotinine-(methyl-D₃)-3'-*O*-glucuronide] and then calculating 3HC-Gluc concentration from the standard curve using Waters' MassLynx software. 3HC-*O*-Gluc and 3HC-*N*-Gluc formation rates were calculated accordingly.

Genotyping Assay

Genomic DNA from subjects who provided HLM samples were genotyped for the UGT2B10 codon 67 polymorphism and UGT2B17 deletion polymorphism as previously described (8, 21).

Statistical Analysis

Kinetic constants were determined by non-linear regression to fit the Michaelis-Menten equation using Prism Version 4.01 software (GraphPad Software, San Diego, CA, USA).

Kinetic values were calculated as a mean of three independent experiments. The rate of 3HC-*N*-Gluc formation in HLM was compared for different *UGT2B10* codon 67 genotypes by Student's *t*-test and by the trend test.

Results

Characterization of 3HC glucuronidation

To analyze glucuronidation activities against 3HC, a rapid UPLC-MS/MS method was developed to quantify 3HC-*N*-Gluc and 3HC-*O*-Gluc simultaneously. As demonstrated by a representative UPLC chromatograph (Figure 2A), two peaks were observed by UPLC in incubations with HLM, with their retention times identical to those of purified 3HC-*N*-Gluc (Figure 2B) and 3HC-*O*-Gluc (Figure 2C) standards at 1.3 and 2.6 min, respectively. Both peaks were sensitive to beta-glucuronidase while only the 2.6 min peak was sensitive to 1M NaOH (results not shown), a pattern similar to that described previously for NNAL-*N*-Gluc and NNAL-*O*-Gluc, respectively (22, 23).

To screen for individual UGT enzyme activity *in vitro*, homogenates of UGT-over-expressing cell lines were used in assays to identify the specific UGTs that exhibited *O*- or *N*-glucuronidation activities against 3HC. While homogenates from UGTs 2B17, 2B7 and 1A9 exhibited the highest activity for formation of 3HC-*O*-Gluc, detectable levels of activity were also observed for UGTs 2B15, 2B10, 2B4, 1A10, 1A7, and 1A4 for 3HC-*O*-Gluc formation (Figure 3, panel A). Due to the low levels of activity observed for these latter enzymes, kinetic analysis could only be performed for homogenates of UGTs 2B17, 2B7 and 1A9-over-expressing cells against 3HC. Representative Michaelis-Menten plots of 3HC-*O*-Gluc formation rate for UGTs 1A9, 2B7 and 2B17, as well as for HLM, are shown in Figure 4 (panel A). UGT2B17 exhibited a 3.7- to 4.2-fold lower K_M and a 3.1- to 8.0-fold higher V_{max}/K_M against 3HC than UGTs 2B7 or 1A9, respectively (Table 1). The average K_M for 3HC-*O*-Gluc formation (26 mM) among HLM from 10 individuals was marginally lower than that observed for UGT2B7 (31 mM) or UGT1A9 (35 mM) but were higher than that observed for UGT2B17 (8.3 mM).

Of the 14 UGTs screened, only UGTs 1A4 and 2B10 exhibited 3HC-*N*-Gluc formation activity (Figure 3, panel B). Representative Michaelis-Menten plots of 3HC-*N*-Gluc formation rate for UGTs 2B10 and 1A4, as well as for HLM, are shown in Figure 4 (panel B). Kinetic analysis demonstrated that homogenates from UGT2B10-over-expressing cells exhibited a K_M (13 mM) for 3HC-*N*-Gluc formation that was similar to HLM (14 mM) and was 4.4-fold lower than that observed for homogenates from UGT1A4-over-expressing cells (Table 2). The V_{max} for UGT2B10-over-expressing cell homogenates was greater than 85-fold higher than that observed for homogenates from UGT1A4-over-expressing cells.

Association between *UGT* genotypes and 3HC glucuronidation activity

A prevalent missense (Asp>Tyr) polymorphism exists for UGT2B10 at codon 67 (8). A HEK293 cell line over-expressing the UGT2B10^{67Tyr} variant has been described previously (8, 16), with normalization of UGT2B10 protein expression performed in the wild-type and variant UGT2B10-over-expressing cell lines after Western blot analysis as previously described (24). Unlike the activity observed for the wild-type UGT2B10^{67Asp}, no glucuronidation activity was observed for the UGT2B10^{67Tyr} variant against 3HC (results not shown). To determine whether the UGT2B10 codon 67 (Asp>Tyr) polymorphism was associated with decreased 3HC-*N*-Gluc formation activity, levels of 3HC-*N*-Gluc formation was examined in a series (n=110) of microsomal specimens prepared from normal human liver tissue from individual subjects. There was a significant ($P<0.05$) trend towards decreased 3HC-*N*-glucuronidation activity against 3HC in HLM from subjects with an

increasing number of the variant UGT2B10^{67Tyr} (UGT2B10*2) allele ($P_{\text{trend}} < 0.01$, Figure 5). There was a significant ($p < 0.001$) 33% decrease in 3HC-*N*-Gluc formation activity in HLM from subjects heterozygous for UGT2B10 (*1/*2) genotype and a significant ($p < 0.001$) 12-fold decrease in 3HC-*N*-Gluc formation activity in HLM from subjects homozygous for UGT2B10 (*2/*2) genotype as compared to subjects with the wild-type UGT2B10 (*1/*1) genotype.

A prevalent whole-gene deletion polymorphism has been reported for UGT2B17 (21, 25). It was previously demonstrated that the homozygous UGT2B17 (*2/*2) deletion genotype is associated with significantly reduced activity for 3HC-*O*-Gluc formation in HLM, suggesting that UGT2B17 is an important contributor to hepatic glucuronidation activity against 3HC (26). To further determine whether the UGT2B17 deletion alters hepatic 3HC glucuronidation activity, kinetic analysis of 3HC-*O*-Gluc formation was performed in HLM from subjects exhibiting either the wild-type *UGT2B17* (*1/*1) genotype ($n=6$) or the homozygous deletion *UGT2B17* (*2/*2) genotype ($n=6$). The mean K_M (15.6 mM) in HLM from subjects with the *UGT2B17* (*1/*1) genotype was 3.1-fold lower ($p < 0.001$) than that observed in HLM from subjects with the *UGT2B17* (*2/*2) genotype (Table 3). No significant difference in V_{max} or V_{max}/K_M was observed between the two HLM groups.

Discussion

Previous studies had identified several UGT enzymes active against 3HC, including UGTs 1A9 and 2B7 which were active in 3HC-*O*-Gluc formation, and UGT1A4 which catalyzed 3HC-*N*-Gluc formation, but UGTs 2B10 and 2B17 were not analyzed in these studies (9, 11). In the present study, it was demonstrated that UGT2B17 was more active against 3HC than other UGTs for 3HC-*O*-Gluc formation. UGT2B17 exhibited a ~3–8-fold higher V_{max}/K_M than the next most active UGTs, 1A9 or 2B7. While there was some variability in the kinetic values of 3HC-*O*-glucuronide formation of individual HLMs after stratification by UGT2B17 genotype, HLM from subjects homozygous for the *UGT2B17* deletion exhibited a significant 3.1-fold increase in K_M for 3HC-*O*-Gluc formation as compared to HLM from subjects wild-type for UGT2B17. This was potentially due to these UGT2B17-deleted HLM approximating the K_M observed for other 3-HC-glucuronidating hepatic UGTs (i.e., 1A9 and 2B7). The variability between HLM specimens could be due to variations in the activity or expression of other 3HC glucuronidating enzymes (i.e., UGTs 1A9 or 2B7), or possibly due to differences in the overall quality of the HLMs. Nevertheless, these data are consistent with previous studies demonstrating an association between *UGT2B17* deletion genotypes and decreased 3HC-*O*-Gluc formation activity in HLM (2) and with reduced 3HC-*O*-Gluc levels in smoker's urine (2).

Previous studies have demonstrated that the hepatic expression of UGT2B17 was found to be 7.1–21-fold lower than UGT2B7 (13, 14). In addition, while the hepatic expression of UGT2B17 was found to be 2.8-fold higher than UGT1A9 in one study (13), its expression was 6.1-fold lower in another study (14). Given the relative activity of each enzyme against 3HC observed in the present study, it is likely, therefore, that all three UGT enzymes play an important role in hepatic 3HC glucuronidation.

The present study is also the first to demonstrate that UGT2B10 is the most active UGT for 3HC-*N*-Gluc formation. It exhibits a nearly 400-fold higher V_{max}/K_M than UGT1A4 and a K_M similar to HLM, suggesting that UGT2B10 is responsible for the majority of the 3HC-*N*-Gluc formation activity in human liver. The important role of UGT2B10 in hepatic 3HC-*N*-Gluc formation is further confirmed by the fact that HLM from subjects with the UGT2B10 (*2/*2) genotype exhibited a 12-fold reduction in 3HC-*N*-Gluc formation activity as compared with that of the wild-type UGT2B10 (*1/*1) genotype. Unfortunately, a

comparison of kinetic parameters for 3HC-*N*-Gluc formation could not be determined in HLM stratified by UGT2B10 genotype because of low overall *N*-glucuronidation activity in the UGT2B10 (*2/*2) HLM. This is consistent with the significantly less active UGT1A4 being the only remaining 3HC-*N*-glucuronidating enzyme in these samples.

Previous studies demonstrated that HLM were capable of making 3HC-*N*-Gluc and that UGT1A4 was the only active enzyme in this process (11). In the present study, UGT2B10 was more active than UGT1A4 for 3HC-*N*-Gluc formation. The importance of UGT2B10 in the hepatic formation of 3HC-*N*-Gluc was implicated by the fact that the K_M of 3HC-*N*-Gluc formation in HLM was very similar to that observed for UGT2B10 *in vitro* and was 4.4-fold lower than that observed for UGT1A4 homogenates. In addition, HLM from subjects homozygous for the UGT2B10*2 allele, which contains a functional knock-out of UGT2B10 activity by the presence of a tyrosine residue at codon 67, exhibits ~9% of the 3HC-*N*-Gluc formation activity as compared to HLM from subjects wild-type for UGT2B10, suggesting that in livers where UGT2B10 is active, UGT1A4 accounts for only 9% of the total 3HC-*N*-Gluc activity. The lack of *in vitro* activity observed for the UGT2B10^{67Tyr} variant is similar to that observed for other substrates including nicotine, cotinine, NNAL and olanzipine (8, 16, 24), and the association between decreased HLM 3HC-*N*-Gluc formation with the *UGT2B10* (*2/*2) genotype is also similar to that observed previously for other substrates (8, 27) and with urinary levels of nicotine and cotinine (2).

While HLM clearly catalyzed the formation of 3HC-*N*-Gluc, with a V_{max}/K_M that was similar and a K_M that was in fact lower than that observed for 3HC-*O*-Gluc formation, it is still unclear why 3HC-*N*-Gluc is not detected in smokers' urine (9, 11), a fact that was confirmed in this laboratory (results not shown). Other *N*-glucuronide products of UGT2B10, including both nicotine-*N*-Gluc and cotinine-*N*-Gluc, are observed at high levels in the urine of smokers (2, 5, 28). It is possible that 3HC-*N*-Gluc is formed *in vivo* but is excreted into the bile rather than urine. Glucuronide conjugates of nicotine, cotinine, and 3HC are the major nicotine metabolites found in the bile of rats after nicotine administration (29). It is also possible that 3HC-*N*-gluc is extensively converted to other as yet unknown metabolite forms via an unknown mechanism. A pharmacokinetic study of 3HC-*N*-Gluc metabolism will be necessary to better address this question.

In summary, the results from the present study demonstrate that UGT2B17 and UGT2B10 are the most active UGT enzymes in the glucuronidation of 3HC to form 3HC-*O*-Gluc and 3HC-*N*-Gluc, respectively. The present study demonstrates that genetic variations in the UGT2B17 and UGT2B10 genes resulting in functional knockouts are associated with significantly reduced activities against 3HC in HLM. While 3HC is an inactive metabolite of nicotine, the importance of the 3HC glucuronidation pathway is magnified since the ratio of 3HC:cotinine has been used extensively as a phenotypic marker for nicotine metabolism and in nicotine intervention trials (30). Since the glucuronidation of 3HC could affect the levels of 3HC in urine, therefore affecting the ratio of 3HC:cotinine, delineation of 3HC glucuronidation pharmacogenetics may prove important when utilizing the 3HC:cotinine ratio as a biomarker for nicotine metabolism and nicotine-related clinical applications.

Acknowledgments

We thank the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center for the tissues used in these studies. The authors also thank the Functional Genomics Core Facility at the Penn State University College of Medicine for DNA genotyping services. These studies were supported by a Dean's Feasibility Grant from Penn State College of Medicine (Chen), two formula grants under the Pennsylvania Department of Health's Health Research Formula Funding Program (SAP4100038715 [Lazarus] and SAP4100038714 [Whitehead]); State of PA, Act 2001-77 – part

of the PA Tobacco Settlement Legislation), and Public Health Service grant R01-DE13158 (Lazarus) from the National Institutes of Health.

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Figure 1. Nicotine metabolism pathway

Shown is a schematic of the major nicotine metabolites found in the urine of smokers, with percentages indicating their relative abundance according to the studies of Chen et al. (2).

Figure 2. Representative chromatograms of LC-MS/MS analysis of *trans*-3'-HC glucuronidation in HLM

Panel A, HLM catalyzes 3HC to form both 3HC-*N* and *O*-Gluc; panel B, 3HC-*O*-Gluc standard; panel C, 3HC-*N*-Gluc standard. The m/z ion transition was recorded in the multiple reaction monitoring mode: m/z 369 > 193 for *trans*-3'-HC glucuronide.

Figure 3. Individual UGT-over-expressing cell homogenate glucuronidation activity against 3HC Panel A, 3HC-O-Gluc; panel B, 3HC-N-Gluc. 3HC-Gluc formation assays were performed with 2 mM 3HC for 1 h at 37°C using 100 mg total cell homogenate protein. The rate of formation of 3HC-Gluc (V) was adjusted per mg of total homogenate protein as determined by BCA assay. ND, no detectable activity.

Figure 4. Representative Michaelis-Menton curves for 3HC-Gluc formation

Representative curves for homogenates from UGT-over-expressing cell lines and HLM are shown. Panel A, 3HC-*O*-Gluc formation; panel B, 3HC-*N*-Gluc formation. 3HC-Gluc formation assays were performed for 1 h at 37°C using 50 mg total cell homogenate protein or 5 mg HLM total protein. The rate of formation of 3HC-Gluc (V) was adjusted per mg of the corresponding UGT protein as determined by Western blot analysis, and per mg total protein as determined by BCA assay.

Figure 5. Rate of 3HC-N-Gluc formation in HLM stratified by UGT2B10 genotype
 Glucuronidation activity assays were performed using 2 mM 3HC and 10 mg of HLM protein and incubated for 1 h at 37°C as described in the Materials and Methods. Columns, mean; bars, SE. Numbers under the x-axis refer to the number of HLM examined for each of the corresponding UGT2B10 genotypes.

Table 1Kinetic analysis of 3HC-*O*-glucuronide formation using UGT-over-expressing cell homogenates.

UGT ^a	K _M (mM)	V _{max} (pmol·min ⁻¹ ·protein ⁻¹) ^b	V _{max} /K _M (nL·min ⁻¹ ·protein ⁻¹) ^b
UGT2B17	8.3 ± 3.5 ^c	4.8 ± 1.3	0.61 ± 0.12 ^d
UGT2B7	31 ± 3.9	3.7 ± 0.51	0.12 ± 0.01
UGT1A9	35 ± 4.5	2.6 ± 0.10	0.075 ± 0.007
HLM (n=10)	26 ± 16	316 ± 211	18 ± 16

^a Cell line glucuronidation reactions were performed using 50 mg of UGT-over-expressing cell homogenate and incubated for 1 h at 37°C. HLM reactions were performed for 10 HLM specimens in independent glucuronidation reactions using 5 mg of total microsomal protein per reaction. Kinetic data are reported as the mean ± SD for three independent experiments.

^b V_{max} and V_{max}/K_M values are adjusted per µg of the corresponding UGT protein in each of the over-expressing cell homogenates as determined by Western blot analysis, or per mg of total microsomal protein for HLM as determined by BCA assay.

^c p<0.005, and

^d p<0.05, compared to UGT1A9 or UGT2B7, as determined by the Student's t-test.

Table 2Kinetic analysis of 3HC-*N*-glucuronide formation using UGT-over-expressing cell homogenates.

UGT ^a	<i>K_M</i> (mM)	<i>V_{max}</i> (pmol·min ⁻¹ ·protein ⁻¹) ^b	<i>V_{max}/K_M</i> (pmol·min ⁻¹ ·protein ⁻¹) ^b
UGT1A4	57 ± 14	3.57 ± 1.2	0.062 ± 0.012
UGT2B10	13 ± 1.6 ^c	309 ± 88	24 ± 3.9 ^d
HLM	14 ± 4.6	258 ± 329	19 ± 22

^aUGT1A4 and UGT2B10 reactions were performed using 50 mg of UGT-over-expressing cell homogenate and incubated for 1 h at 37°C. HLM reactions were performed for 10 HLM specimens in independent glucuronidation reactions using 5 mg of total microsomal protein per reaction. Kinetic data are reported as mean ± SD for three independent experiments.

^b*V_{max}* and *V_{max}/K_M* values were adjusted per µg of the corresponding UGT protein in each of the over-expressing cell homogenates as determined by Western blot analysis, or per mg of total microsomal protein for HLM as determined by BCA assay.

^c*p* < 0.01, and

^d*p* < 0.05, compared to UGT1A4, as determined by the Student's *t*-test.

Table 3Kinetic analysis of 3HC-*O*-glucuronide formation in HLM stratified by UGT2B17 deletion genotypes.

UGT2B17 genotype ^a	K_M (mM)	V_{max} (pmol·min ⁻¹ ·protein ⁻¹) ^b	V_{max}/K_M (pmol·min ⁻¹ ·protein ⁻¹) ^b
*1/*1	15.6 ± 11.4	331 ± 272	36.7 ± 35.6
*2/*2	48.4 ± 10.7 ^c	447 ± 210	9.1 ± 4.3

^aHLM reactions were performed using 5 mg of total microsomal protein and incubated for 1 h at 37°C. Kinetic data are reported as mean ± SD for six subjects for each UGT2B17 genotype group.

^b V_{max} and V_{max}/K_M values were adjusted per mg of total microsomal protein for HLM as determined by BCA assay.

^c $p < 0.001$ compared to HLM from subjects with the UGT2B17 (*1/*1) genotype, as determined by the Student's *t*-test.