Limited influence of *UGT1A1**28 and no effect of *UGT2B7**2 polymorphisms on *UGT1A1* or *UGT2B7* activities and protein expression in human liver microsomes

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What is already known about this subject

- The *UGT1A1**28 polymorphism is known to reduce *UGT1A1* enzyme activities, via an extra TA repeat in the promoter.
- However, a gap exists with regard to a comprehensive assessment of the influence of this genotype on variability in enzyme activity.
- There is equivocal evidence on the functional relevance of the *UGT2B7**2 polymorphism on *UGT2B7* enzyme activities.

What this study adds

- Using comprehensive approaches to measure enzyme activities and protein expression levels, the *UGT1A1**28 polymorphism is shown to contribute to only 40% of the variability in enzyme activities for *UGT1A1*.
- A novel, nonproprietary method for genotyping *UGT1A1**28 is provided.
- Definitive evidence is provided to conclude there is no effect of the *UGT2B7**2 polymorphism on zidovudine glucuronidation activity.

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Aims

UGT1A1 and *UGT2B7* are enzymes that commonly contribute to drug glucuronidation. Since genetic factors have been suggested to contribute to variability in activities and expression levels of these enzymes, a quantitative assessment of the influence of the major genotypes (*UGT1A1**28 or *UGT2B7**2) on enzyme activities was conducted.

Methods

Using a bank of microsomal samples from 59 human livers, the effect of *UGT1A1**28 or *UGT2B7**2 polymorphisms were investigated on rates of estradiol 3-glucuronidation (a marker of *UGT1A1* enzyme activity) or zidovudine glucuronidation (a marker of *UGT2B7* enzyme activity) and levels of immunoreactive protein for each enzyme. Glucuronidation rates for both enzymes were measured at K_m/S_{50} and 10 times K_m/S_{50} concentrations.

Results

UGT1A1 and *UGT2B7* enzyme activities varied up to 16-fold and sixfold, respectively. Rates at K_m/S_{50} concentration closely correlated with rates at 10 times K_m/S_{50} concentration for both enzymes (but not at 1/10th K_m for *UGT2B7*). Enzyme activities correlated with relative levels of immunoreactive protein for *UGT1A1* and *UGT2B7*. Furthermore, rates of zidovudine glucuronidation correlated well with rates of glucuronidation of the *UGT2B7* substrate gemcabene, but did not correlate with *UGT1A1* enzyme activities. For the *UGT1A1**28 polymorphism, consistent with levels of *UGT1A1* immunoreactive protein, mean *UGT1A1* activity was 2.5- and 3.2-fold lower for TA_6/TA_7 ($P < 0.05$) and TA_7/TA_7 ($P < 0.001$) genotypes in comparison with the TA_6/TA_6 genotype.

Conclusions

Relative to the observed 16-fold variability in *UGT1A1* activity, these data indicate only a partial (approximately 40%) contribution of the *UGT1A1**28 polymorphism to variability of interindividual differences in *UGT1A1* enzyme activity. For the *UGT2B7**2 polymorphism, genotype had no influence on immunoreactive *UGT2B7* protein or the rate of 3'-azido-3'-deoxythymidine glucuronidation.

Introduction

For the top 200 drugs prescribed in the USA in 2003, UGT1A1 and UGT2B7 are among the most commonly listed enzymes contributing to drug glucuronidation [1]. Substrates of UGT1A1 include bilirubin, ethinylestradiol, buprenorphine, paracetamol and the irinotecan metabolite SN-38, whereas UGT2B7 substrates include opioids, zidovudine, nonsteroidal anti-inflammatory drugs, epirubicin, catechol oestrogens, retinoids and fatty acids [1].

Factors known to contribute to variability in expression levels or enzyme activities for UGT1A1 include co-administered drugs [2], disease state [3] and convincing evidence of a functional promoter polymorphism known as *UGT1A1**28 [4]. The wild-type *UGT1A1* allele (TA₆) has six thymine adenine (TA) repeats in the TATA box region of the *UGT1A1* promoter. The variant alleles consists of five, seven or eight TA repeats, are known to be associated with decreased gene expression *in vitro* and are responsible for Gilbert's syndrome, characterized by mild hyperbilirubinaemia. The above-mentioned genetic information prompted our further investigation of *UGT1A1**28 polymorphism effects on variability in UGT1A1 enzyme activities in a bank of 59 human livers. No commercially available reagents were available to perform this assay in a high-throughput manner, therefore we developed a novel method of detecting the *UGT1A1**28 (TA₇), *UGT1A1**36 (TA₅) and *UGT1A1**37 (TA₈) mutations for the Sequenom, Inc. (San Diego, CA, USA) genotyping platform.

For UGT2B7, co-administered drugs may cause small changes in enzyme activity [1]. There is equivocal evidence for the functional relevance of a polymorphism that results in either a histidine (encoded by *UGT2B7**1) or tyrosine (encoded by *UGT2B7**2) at amino acid 268 (H268Y) of the protein. *In vitro* experiments indicate a twofold higher activity in glucuronidation of 4-hydroxestrone or 4-hydroxyestradiol by the *UGT2B7**2 variant compared with *UGT2B7**1 [5]. However, in two studies using genotyped liver microsomal samples, the *UGT2B7**2 polymorphism had no effect on rates of zidovudine or morphine-3-glucuronidation [6, 7]. The reason for the aforementioned differences has not been extensively studied. The above-mentioned genetic information prompted our further investigation of *UGT2B7**2 polymorphism effects on variability in UGT2B7 enzyme activities in a bank of 59 human livers.

The current investigation was conducted to assess quantitatively the contributions of these major genotype polymorphisms of *UGT1A1* and *UGT2B7* genes to variability in enzyme activities and expression levels of

immunoreactive protein. An understanding of the influence of these genotypes would allow an estimation of the *in vivo* effects of these genotypes on hepatic metabolism of drugs glucuronidated by these enzymes. In order to achieve this aim, microsomes from a bank of 59 genotyped human livers were phenotyped for these activities. Estradiol-3 glucuronidation (E3G) was used as a marker of UGT1A1 activity, and zidovudine glucuronidation was used as a marker of UGT2B7 activity [7, 8]. These are well-established probe substrates for their respective enzymes. In order to assess the influence of substrate concentration on variability in enzyme activity, substrate concentrations were selected at K_m/S_{50} and 10 times K_m/S_{50} for both enzymes, and at 1/10th K_m for UGT2B7. Expression levels of immunoreactive protein were also measured using antibodies to UGT1A1 and UGT2B7.

Materials and methods

Chemicals

Pooled (mixed gender, $N=59$) and individual human liver microsomes (HLM) (mixed gender, $N=59$) were obtained from BD Gentest (Woburn, MA, USA) with no donors having liver cirrhosis or cancer. Zidovudine glucuronide (AZTG) was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Unless otherwise stated, all other chemicals were commercially available and of analytical grade from Sigma-Aldrich (St Louis, MO, USA).

*UGT1A1**28 genotype determination

The *UGT1A1* promoter (TA₅₋₈) sequence was determined using a novel high-throughput genotyping protocol. Standard Sequenom, Inc. MassCleave methodology using the C transcription cocktail reaction for the reverse strand was used to cleave the *UGT1A1* promoter sequence. Products cleaved with the C reaction generated sufficiently large and resolvable products (~632 D difference for each TA repeat) containing *UGT1A1* TATA sequence that were subsequently used in a standard genotyping reaction. The *UGT1A1* promoter region was polymerase chain reaction (PCR)-amplified from human genomic DNA using primers designed to incorporate the TA₇ promoter sequence to the reverse strand. The primers (forward 5'-aggaagagag TTT TTA TAG TCA CGT GAC ACA GTC AAA C-3', reverse 5'-cagtaatacgcactcactatagggaagcct CTT TGC TCC TGC CAG AGG TT-3') modified from [9] were used to yield a 148-bp product. Lower case nucleotides in the primer sequences indicate a 10-bp tag used for the forward primer and the T₇ promoter sequence for the reverse.

PCR reactions were carried out in a total volume of 20 µl using 6 pmol of each primer, 200 µM deoxyribonucleotide triphosphate (Invitrogen, Carlsbad, CA, USA), 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1.5 mM MgCl₂, 10× AmpliTaq Gold buffer supplied with the enzyme (final concentration 1×) and 20 ng of genomic DNA. The reaction mix was preactivated for 15 min at 95°C. PCR consisted of 45 cycles of 95°C for 20 s, 56°C for 30 s and 72°C for 1 min followed by 72°C for 3 min. Reactions were then split into triplicate wells (5 µl each). Unincorporated deoxyribonucleotide triphosphates were dephosphorylated by adding 1.7 µl H₂O and 0.3 U shrimp alkaline phosphatase (SAP) (Sequenom, Inc.) The reaction was incubated at 37°C for 20 min and heat-inactivated at 85°C for 5 min.

For *in vitro* transcription, 2 µl of the PCR reaction was directly used as template in a 4-µl total reaction volume. Twenty units of R&DNA polymerase (Epicentre, Madison, WI, USA) ribonucleotides to a final concentration of 1 mM, and deoxyribonucleotide triphosphate to 2.5 mM were added and the mixture was incubated for 2 h at 37°C; other components in the reaction were as recommended by the supplier. Following *in vitro* transcription, RNase A was added (final concentration 0.08 mg ml⁻¹) and the reaction incubated for 1 h at 37°C. The PCR/*in vitro* transcription mixture was then further diluted with H₂O to a final volume of 27 µl. Conditioning of the phosphate backbone prior to matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) was achieved by the addition of 6 mg CLEAN Resin (Sequenom Inc.) [4].

*UGT2B7*2 genotype determination*

The *UGT2B7*2* region was PCR-amplified from human genomic DNA using primers that incorporate the exon 2 sequence and flank the histidine to tyrosine at amino acid 268. The primers (forward 5'-acgttgatg GGG AAA GCT GAC GTA TGG CTT ATT-3', reverse 5'-acgttgatg TCC AAC AAA ATC AAC ATT TGG TAA GAG-3'), modified from Ref. [10], were used to yield a 104-bp product. Lower case nucleotides in the primer sequences indicate the 10-bp tag used for both primers.

The PCR reactions were carried out in a total volume of 20 µl using 1.5 pmol of each primer, 50 µM deoxyribonucleotide triphosphate (Invitrogen), 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 1.5 mM MgCl₂, buffer supplied with the enzyme (final concentration 1×) and 20 ng of genomic DNA. The reaction mix was preactivated for 15 min at 95°C. PCR consisted of 45 cycles of 95°C for 20 s, 56°C for 30 s and

72°C for 1 min followed by 72°C for 3 min. Reactions were then split into duplicate wells (5 µl each). Unincorporated deoxyribonucleotide triphosphates were dephosphorylated by adding 1.53 µl H₂O, 0.17 µl of hME buffer (Sequenom, Inc.) and 0.3 U SAP. The reaction was incubated at 37°C for 20 min and heat-inactivated at 85°C for 5 min.

The PCR/SAP reaction (7 µl) was directly used as template in a MassEXTEND reaction. The total volume of each reaction was 9 µl, including template extension primer (5'-CCT GGA ATT TTC AGT TTC C-3'), Thermosequenase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the termination mix (deoxyadenosine 5'-triphosphate, dideoxycytidine 5'-triphosphate, dideoxyguanosine 5'-triphosphate and dideoxythymidine 5'-triphosphate). Allele-specific extended products were obtained under standard Sequenom MassEXTEND cycling conditions (94°C for 2 min, followed by 99 cycles of 94°C for 5 s, 52°C for 5 s and 72°C for 5 s). Conditioning of the phosphate backbone prior to MALDI-TOF-MS was achieved by the addition of 3 mg CLEAN Resin (Sequenom Inc.) [4].

DNA isolation

Genomic DNA was isolated using the QIAamp DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Briefly, 25 mg of liver was placed in a 1.5-ml centrifuge tube and 180 µl Qiagen Buffer ATL was added. Proteinase K (12 mAU) was added to each tube, vortexed and incubated at 55°C overnight. After incubation, 400 µl of Buffer AL-ethanol mixture was added to the sample and vortexed vigorously. The mixture was transferred to a DNeasy Mini Spin column and centrifuged at 6000 g for 1 min. The column was transferred to a fresh tube, 500 µl Buffer AW1 was added, and centrifuged again at 6000 g for 1 min. The column was transferred to a fresh tube, 500 µl Buffer AW2 was added, and centrifuged at 20 000 g for 3 min. The column was transferred to another fresh tube, 200 µl Buffer AE was added, and centrifuged at 6000 g for 1 min.

Mass spectrometry determination of genotype

Genotyping was performed under Good Laboratory Practice guidelines and all assays had been previously validated. In-house samples, validated by nucleic acid sequencing, were used for quality control. Fifteen nanolitres of the cleavage reaction or MassEXTEND was robotically dispensed (Nanodispenser; Sequenom, Inc.) onto silicon chips preloaded with matrix (SpectroCHIP bioarrays; Sequenom, Inc.). Mass spectra were collected using the genotyping software provided on the Sequenom Biflex platform (MassARRAY Typer, Version

3.1.4). MassARRAY Typer software performed genotype calling using a set of digital filters optimized for mass spectra of oligonucleotides. The distribution of *UGT1A1* and *UGT2B7* genotypes was in Hardy-Weinberg equilibrium (HWE).

Human liver microsomal samples

The human liver bank consisted of 59 livers from individuals 18–65 years of age, both male ($n = 33$) and female ($n = 26$) and of White ($n = 52$), Hispanic ($n = 4$) and African-American ($n = 3$) ethnic origins. The initial protein concentrations of HLMs varied with each of the 59 liver samples, ranging from 10.5 mg ml^{-1} to 28.2 mg ml^{-1} . The initial protein concentration of the pooled human liver microsomal protein was 20.4 mg ml^{-1} .

Phenotyping: UGT1A1-catalysed estradiol-3-glucuronidation

Assay optimization led to the following conditions: HLMs (0.5 mg ml^{-1}), Tris-HCl buffer (50 mM , pH 7.1) and alamethicin ($50 \text{ } \mu\text{g mg}^{-1}$ of protein) were mixed and preincubated on ice for 15 min. This was followed by the addition of MgCl_2 (5 mM) and β -estradiol (in 0.5% dimethylsulphoxide). S_{50} and V_{max} values were determined using pooled human liver microsomal samples ($N = 59$). The rates of estradiol glucuronidation were determined in triplicate at substrate S_{50} ($25 \text{ } \mu\text{M}$) and at a UGT saturating concentration of $10 \times S_{50}$ ($250 \text{ } \mu\text{M}$). After a 5-min preincubation period in a shaking water bath (37°C), the reaction was started by the addition of the cofactor uridine diphosphoglucuronic acid (UDPGA) (5 mM) in a final incubation volume of $200 \text{ } \mu\text{l}$. After 30 min, the reaction was quenched by addition of $50 \text{ } \mu\text{l}$ formic acid ($25\% \text{ v/v}$). Subsequently, $10 \text{ } \mu\text{l}$ of α -naphthylglucuronide (internal standard, $1 \text{ } \mu\text{M}$) was added and tubes were centrifuged at $14\,000 \text{ r.p.m.}$ ($15\,000 \text{ g}$) for 10 min. The resulting supernatant was removed and analysed by high-performance liquid chromatography with ultraviolet detector (HPLC-UV) to quantify E3G.

HPLC-UV analyses for quantification of estradiol-3-glucuronide were performed on a Perkin-Elmer Series 200 autosampler and LC pump equipped with a Perkin Elmer 785A UV/Vis detector and NCI 900 network chromatography interface (Perkin-Elmer, Boston, MA, USA), utilizing an Agilent Zorbax SB-C18, $5 \text{ } \mu\text{m}$, $150 \times 4.6 \text{ mm}$ HPLC column (Agilent Technologies, Palo Alto, CA, USA). The mobile phase solution A was H_2O [0.1% trifluoroacetic acid (TFA)] and solution B was acetonitrile: $\text{H}_2\text{O}/0.1\%$ TFA ($90 : 10, \text{ } \%$ v/v). Initial conditions were $71\% \text{ A}/29\% \text{ B}$ at a flow rate of 1.0 ml min^{-1} followed by a linear gradient from 29

to $60\% \text{ B}$ in 8 min, followed by a step gradient to $100\% \text{ B}$ for 1 min, and then a 4-min re-equilibration at $29\% \text{ B}$. The retention times for α -naphthylglucuronide, estradiol-3-glucuronide, estradiol-17-glucuronide and estradiol were approximately 4.5, 5.2, 6.2 and 10.2 min , respectively. A standard curve was generated ranging from 12 ng ml^{-1} to $100 \text{ } \mu\text{g ml}^{-1}$. Standard curve correlation coefficients, using linear regression (r^2) were ≥ 0.99 and the lower limit of quantification was 12 ng ml^{-1} . Intersubject percent coefficient of variation ($\% \text{ CV}$) was <10 and interday $\% \text{ CV}$ was <15 .

UGT2B7-catalysed 3'-azido-3'-deoxythymidine glucuronidation assay

Assay optimization led to the following conditions: HLMs total protein (0.25 mg ml^{-1}), Tris buffer (50 mM , pH 7.1) and alamethicin ($50 \text{ } \mu\text{g mg}^{-1}$ of protein) were mixed and preincubated on ice for 15 min. This was followed by the addition of MgCl_2 (5 mM) and $3'$ -azido- $3'$ -deoxythymidine (AZT) at K_m (1.5 mM), or $1/10\text{th } K_m$ (0.15 mM), or $10 \times K_m$ (15 mM). K_m values were determined using pooled human liver microsomal samples ($N = 59$). After a 5-min preincubation period in a shaking water bath (37°C), the reaction was started by the addition of UDPGA (10 mM) in a final incubation volume of $200 \text{ } \mu\text{l}$. After 30 min, the reaction was quenched by addition of $100 \text{ } \mu\text{l}$ sample to $400 \text{ } \mu\text{l}$ of acetonitrile containing 3-acetamidophenol (internal standard, $7 \text{ } \mu\text{g ml}^{-1}$) or $50 \text{ } \mu\text{l}$ of acetonitrile containing $112 \text{ } \mu\text{g ml}^{-1}$ 3-acetamidophenol to $200 \text{ } \mu\text{l}$ of sample. The samples were then centrifuged at $14\,000 \text{ r.p.m.}$ ($15\,000 \text{ g}$) for 10 min, supernatants removed, mixtures dried under N_2 and reconstituted in mobile phase consisting of $84\% \text{ } 20 \text{ mM}$ potassium phosphate buffer (pH 2.2) and $16\% \text{ acetonitrile}$. The samples that were quenched using $50 \text{ } \mu\text{l}$ acetonitrile were not dried down and reconstituted with no observed difference in chromatography from those that were dried down. Experiments were performed in triplicate on two separate days. Three substrate concentrations (K_m , $K_m/10$ and $10 \times K_m$) were used to investigate relative contributions of UGT2B7 to AZT glucuronidation at different nonsaturating conditions and under saturating conditions.

HPLC-UV analyses for quantification of AZTG were performed by injecting $50 \text{ } \mu\text{l}$ of supernatant on a Phenomenex Aqua, $5 \text{ } \mu\text{m}$, C18, $150 \times 4.6 \text{ mm}$ HPLC column (Phenomenex, Torrance, CA, USA). The HPLC system consisted of an Agilent Series 1100 (Agilent Technologies), equipped with a Perkin-Elmer Series 200 autosampler and LC pump, and a Perkin Elmer 785A UV/Vis detector (Perkin-Elmer). The mobile phase solution A was 20 mM phosphate buffer (pH 2.2) and

solution B was acetonitrile. Initial conditions were 84% A/16% B at a flow rate of 1.0 ml min^{-1} , followed by a linear gradient from 16 to 65% B in 7.1 min and a 4.9-min re-equilibration at 16% B. The retention times for AZTG, 3-acetamidophenol and AZT were approximately 4.1, 5.0 and 5.9 min, respectively. A standard curve was generated ranging from 0.25 ng ml^{-1} to $50 \text{ } \mu\text{g ml}^{-1}$. Standard curve correlation coefficients, using linear regression, were ≥ 0.99 . Intersubject % CV was <10 and interday percent (% CV) was <15 .

Western blot analysis for UGT protein

The relative content of UGT1A1 and UGT2B7 protein in hepatic microsomes from the liver bank was determined by adapting an immunoblotting method previously described [7]. Briefly, $15 \text{ } \mu\text{g}$ of microsomal protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a Criterion™ XT 10% Bis-Tris gel (BioRad, Hercules, CA, USA). Proteins were then electrophoretically transferred to a polyvinyl difluoride membrane (BioRad), blocked with 5% powdered nonfat milk in Tris-buffered saline (TBS)–Tween (0.15 M NaCl , 0.04 M Tris , pH 7.7, and 0.1% Tween 20) and then incubated in TBS–Tween/0.5% milk containing a 1 : 500 dilution of a polyclonal antipeptide UGT1A1 or UGT2B7 antibody (WB-UGT1A1 and WB-UGT2B7; BD Biosciences, Woburn, MA, USA). After washing TBS–Tween, the blots were incubated in a 1 : 500 dilution of horseradish peroxidase-conjugated secondary antibody (BD Biosciences) and washed. Chemiluminescence reagent was applied (LumiGLO; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at room temperature for 1–2 min. Blots were imaged using a Kodak X-OMAT 2000 A processor (Eastman Kodak Co., Rochester, NY, USA), and bands quantified by densitometry using the Gel Doc 2000 Transilluminator and Quantify One Analysis Software (BioRad). Each blot contained a standard curve generated by using serial dilutions of recombinant UGT1A1 or UGT2B7 and control samples. Signal was linear with respect to amount of protein (10–1000 pg). Relative amounts of protein from each liver bank sample were measured against the relevant recombinant UGT standard curve.

Data analysis

Correlation analyses, tests for normality and ANOVA comparisons were performed using GraphPad Prism version 3.03 for Windows (GraphPad Software, San Diego, CA, USA). Analyses of genotype–phenotype relationship for UGT1A1 and UGT2B7 were first carried out using analysis of variance (ANOVA). Enzyme

activities of variant genotypes were compared with that of wild-type homozygous genotype (e.g. TA_6/TA_6 for *UGT1A1**28) using Dunnett's test, which provides appropriate adjustment for multiple comparisons. In addition, rank-based nonparametric statistical method was used to model the genotype–phenotype relationship. ANOVA, Dunnett's test and nonparametric method were carried out using R™ software (<http://www.r-project.org>). ANOVA comparisons of the contribution of the *UGT1A1**28 allele on enzyme activity were performed using JMP version 5.1.1 (SAS Institute Inc., Cary, NC, USA).

Results

*UGT1A1**28 and *UGT2B7**2 genotypes

Genotype frequencies were consistent with previously reported studies [7, 11–14]. The distribution of *UGT1A1* and *UGT2B7* genotypes was in HWE. The genotype frequencies in the *UGT1A1* promoter for the *UGT1A1**28 polymorphism were 0.63 for TA_6 (six TA repeats) and 0.34 for TA_7 (seven TA repeats), respectively. Overall, 38% of the liver bank were TA_6/TA_6 wild type, 48% were TA_6/TA_7 heterozygotes and 9% were TA_7/TA_7 homozygous variants. There was only one sample each with the TA_5/TA_6 , TA_5/TA_7 , and TA_6/TA_8 genotypes, thus they were not included in the final analysis. For the *UGT2B7* gene, the frequency of the *1 and *2 genotypes were 0.49 and 0.51, respectively.

Enzyme activities, protein expression levels and influence of genotype for *UGT1A1*

Kinetic analysis of E3G in pooled HLMs best fit a homotropic activation model ($n = 1.8$) with an apparent S_{50} of $25.6 \pm 3.22 \text{ } \mu\text{M}$ with a V_{max} of $1.70 \pm 0.056 \text{ nmol min}^{-1} \text{ mg}^{-1}$. Enzyme activities were not normally distributed (Figure 1A) and E3G rates at S_{50} and $10 \times S_{50}$ ranged from 0.183 to 1.77 (9.7-fold variability) and 0.287 to 4.62 (16-fold variability) $\text{nmol min}^{-1} \text{ mg}^{-1}$ of protein, respectively. E3G rates at S_{50} and $10 \times S_{50}$ were significantly correlated ($r^2 = 0.853$, $P < 0.0001$, Figure 1B), suggesting that the same enzyme, or coordinately expressed enzymes, contributes to E3G at these two substrate concentrations. UGT1A1 enzyme activities also correlated with UGT1A1 protein content ($r^2 = 0.523$, $P < 0.0001$, Figure 1C, S_{50} concentration). A representative Western blot and corresponding enzyme activities are shown in Figure 1D.

For genotype–phenotype correlations statistical analyses were performed on (i) all liver samples and (ii) White liver samples alone: conclusions on genotype–phenotype correlations were identical for the two

Figure 1

(A) Distribution of individual UGT1A1-catalysed estradiol-3-glucuronidation rates at S_{50} concentration (25 μM) in human microsomes from 59 livers. Data were not normally distributed ($P=0.006$, Kruskal–Wallis normality test). (B) Correlation analysis of UGT1A1-catalysed estradiol-3-glucuronidation rates at S_{50} (25 μM) and $10\times S_{50}$ (250 μM) concentrations of substrate. (C) Correlation analysis of relative UGT1A1 protein content and UGT1A1-catalysed estradiol-3-glucuronidation at S_{50} (25 μM). Rates at the two substrate concentrations were highly correlated ($r^2=0.853$, $P<0.0001$, $n=59$). Rates at S_{50} concentration also correlated well with protein content ($r^2=0.523$, $P<0.0001$, $n=52$). (D) Immunoblot of relative UGT1A1 protein content in individual human liver microsomes compared with a dilution of 10–1000 pg UGT1A1 Supersomes™. The empty lanes were not loaded with protein

analyses. Mean rates of E3G activity for TA_7/TA_7 homozygotes were 2.5-fold ($P<0.005$) and 3.2-fold ($P<0.001$, Figure 2A,B) lower compared with TA_6/TA_6 homozygotes at S_{50} and at $10\times S_{50}$, respectively. Mean rates of E3G activity for TA_6/TA_7 heterozygotes were ~ 1.3 -fold lower compared with TA_6/TA_6 homozygotes at S_{50} and at $10\times S_{50}$ ($P<0.05$, Figure 2A,B). There were no observed significant differences

between homozygous wild types (TA_6/TA_6) and homozygous variants (TA_7/TA_7) between the selected substrate concentrations (Table 1). Results from ANOVA concluded 37% ($P<0.0001$) contribution of the *UGT1A1**28 genotype to overall variability in UGT1A1 enzyme activities at S_{50} concentration and 40% contribution ($P<0.0003$) at 10 times S_{50} concentration. Additionally, ANOVA analysis showed no sex differences

($P > 0.05$) in E3G glucuronidation rates. Consistent with UGT1A1*28 genotype effects on enzyme activities, levels of immunoreactive protein were twofold and

threefold lower in heterozygotes (TA₆/TA₇) and homozygous variants (TA₇/TA₇), respectively, compared with homozygous wild-type (TA₆/TA₆) individuals (data not shown).

Figure 2
Influence of genotype on UGT1A1-catalysed mean glucuronidation rate at (A) S_{50} (25 μ M) and (B) $10 \times S_{50}$ (250 μ M) substrate concentrations. Mean enzyme activities for the TA₆ genotype were up to 3.2-fold lower for the TA₇/TA₇ genotype compared with the TA₆/TA₆ genotype.
* $P < 0.05$; ** $P < 0.01$ (mean $\pm$ SE)

Enzyme activities, protein expression levels and influence of genotype for UGT2B7

The kinetics of UGT2B7-catalysed zidovudine glucuronidation (AZTG), in pooled HLMs, best fit a model followed by Michaelis–Menten kinetics with an apparent K_m of 1.60 ± 0.10 mM and V_{max} of 23.7 ± 0.439 nmol min⁻¹ mg⁻¹. The AZTG rates at K_m ranged from 0.57 to 3.31 nmol min⁻¹ mg⁻¹ of protein (5.8-fold range between lowest and highest activity), showing a normal distribution for this population (Figure 3A). At $10 \times K_m$, AZTG rates ranged from 1.10 to 5.14 nmol min⁻¹ mg⁻¹ of protein (4.7-fold range in activities). Correlation analysis of AZTG rates at K_m concentration of substrate correlated poorly with AZTG rates at 1/10th of K_m ($r^2 = 0.451$, $P < 0.0001$, data not shown), but correlated well at $10 \times K_m$ ($r^2 = 0.770$, $P < 0.0001$, Figure 3B). Levels of immunoreactive protein correlated with UGT2B7 protein content at K_m ($r^2 = 0.513$, $P < 0.0001$, Figure 3C). In a further experiment, AZT glucuronidation rates correlated well with glucuronidation rates for gemcabene ($r^2 = 0.731$, $P < 0.0001$, $n = 22$ samples of HLMs, data not shown), another substrate known to be metabolized by UGT2B7 [15]. Representative blots and corresponding enzyme activities are shown in Figure 3D. E3G activities did not correlate with AZTG activities at either K_m ($r^2 = 0.008$, $P > 0.1$) or $10 \times K_m$ ($r^2 = 0.012$, $P > 0.10$) concentrations of AZT, or with rates of gemcabene glucuronidation ($r^2 = 0.00009$, $P > 0.10$, $n = 22$).

Genotype had no influence on AZTG rates (Figure 4A,B, Table 2) or levels of immunoreactive UGT2B7 protein. There were no sex differences observed for AZTG activity ($P > 0.05$, data not shown).

Table 1

Influence of UGT1A1*28 genotype on estradiol-3 glucuronidation activity in human liver microsomes (nmol min⁻¹ mg⁻¹ protein, mean $\pm$ SE (N))

Genotype	6/6	6/7	7/7
25 μ M estradiol (S_{50})	0.941 $\pm$ 0.064 (21)	0.718 $\pm$ 0.063 (27)*	0.383 $\pm$ 0.097 (5)**
250 μ M estradiol ($10 \times S_{50}$)	1.63 $\pm$ 0.138 (21)	1.22 $\pm$ 0.130 (27)*	0.534 $\pm$ 0.112 (5)**

* $P < 0.05$, compared with 6/6. ** $P < 0.01$, compared with 6/6.

Figure 3

(A) Distribution of individual UGT2B7-catalysed 3'-azido-3'-deoxythymidine (AZT) glucuronidation rates at K_m (1.5 mM) in human microsomes from 59 livers. Data were normally distributed. (B) Correlation analysis of UGT2B7-catalysed AZT glucuronidation at K_m (1.5 mM) and $10 \times K_m$ (15 mM). (C) Correlation analysis of relative UGT2B7 protein content and UGT2B7-catalysed AZT glucuronidation at K_m (1.5 mM). Rates at the two substrate concentrations correlated well ($r^2 = 0.770$, $P < 0.0001$, $n = 59$). Rates at K_m concentration also correlated well with protein content ($r^2 = 0.513$, $P < 0.0001$, $n = 50$). (D) Immunoblot of relative UGT2B7 protein content in individual human liver microsomes compared with a dilution of 10–1000 pg UGT2B7 Supersomes™

Discussion

The data in this study demonstrate partial (37–40%) contribution of the *UGT1A1**28 polymorphism to the overall observed variability in UGT1A1 enzyme activity in a bank of human liver microsomal samples. Further, this contribution is explained by reduced expression of UGT1A1 protein in those individuals carrying an extra TA repeat in the *UGT1A1* promoter. A novel method is described using the Sequenom platform for genotyping

the *UGT1A1**28 polymorphism. There was no observed effect of the *UGT2B7**2 polymorphism on UGT2B7 enzyme activity or expression levels of immunoreactive protein.

The experimental approach employed for phenotyping this bank of HLMs was designed to investigate the contribution of substrate concentration to variability in enzyme activities. The high degree of correlation in enzyme activities at K_m/S_{50} and $10 \times K_m/S_{50}$ concentrations

indicates the suitability of either concentration for phenotyping using E3G and AZTG as respective probes of UGT1A1 and UGT2B7 activity in HLMs. Furthermore, correlation of AZTG rates with gemcabene glucuronida-

tion rates provides further confidence that the activities measured accurately reflect levels of active UGT2B7 enzyme at K_m and $10\times K_m$ concentration. However, the observation that rates of AZTG at $1/10$ th K_m concentrations of AZT did not correlate with rates at K_m concentration suggests the contribution of enzyme(s) other than UGT2B7, possibly UGT2B4 [7], to AZT glucuronidation in this situation.

The observed *in vitro* variability in UGT1A1 activity (up to 16-fold) is consistent with previous observations – variability for bilirubin (at substrate concentrations 12-fold greater than S_{50}) and SN-38 ($\sim K_m$), known UGT1A1 substrates, have been shown to be ≥ 17 -fold in a bank of 25 livers [13, 14]. Fisher *et al.* have shown a slightly higher (30-fold) variability in enzyme activities in a bank of livers ($n = 21$) at saturating concentrations of estradiol [8]. The observation of an approximate threefold decrease in mean UGT1A1 activity in variant TA_7/TA_7 homozygotes compared with TA_6/TA_6 homozygotes is consistent with observed effects *in vivo*. The ratio of the irinotecan metabolite SN-38 (primarily cleared by UGT1A1) to SN-38 glucuronide has been reported to be threefold higher in TA_6/TA_6 homozygotes compared and TA_7/TA_7 homozygotes in two separate studies [16, 17].

In patients, *UGT1A9* genotype may also influence SN-38 glucuronide levels, although the molecular basis to explain this finding is not fully understood [18]. This is consistent with the assumptions of the well-stirred model of hepatic metabolism: hepatic clearance would be expected to be equal to or less than threefold different between homozygous wild types and homozygous variant alleles, depending on the rate of intrinsic clearance for the UGT1A1 substrate in question [19]. As well as influence on rates of drug clearance, variability in UGT1A1 enzyme activity may also have implications for carcinogen detoxification, as demonstrated by reduced glucuronidation of benzo(a)pyrene-7, 8-dihydrodiol in liver microsomes from subjects with the TA_7/TA_7 genotype [20].

Figure 4
Influence of genotype on UGT2B7-catalysed mean glucuronidation rate at (A) K_m (1.5 mM) and (B) $10\times K_m$ (15 mM) substrate concentrations (mean $\pm$ SE). There was no statistically significant effect of the *UGT2B7**2 polymorphism on the rate of 3'-azido-3'-deoxythymidine glucuronidation between the different genotypes

	*1/*1	*1/*2	*2/*2
1.5 mM (K_m)	1.54 $\pm$ 0.160 (13)	1.65 $\pm$ 0.099 (14)	1.45 $\pm$ 0.135 (27)
15 mM ($10\times K_m$)	2.45 $\pm$ 0.278 (13)	2.69 $\pm$ 0.176 (14)	2.24 $\pm$ 0.240 (27)

No statistically significant differences were observed among genotypes compared with *1/*1.

Table 2

Influence of *UGT2B7**2 genotype on zidovudine glucuronide activity in human liver microsomes (nmol min⁻¹ mg⁻¹ protein, mean $\pm$ SE (N))

UGT2B7 is the major determinant of clearance for epirubicin [21], and gemcabene [15], and has recently been implicated as a major contributor to lamotrigine clearance [22]. Variability in UGT2B7 enzyme activity *in vitro* in this study compares similarly with previously published studies: Court *et al.* [7] have shown variability in enzyme activity in a bank of 54 livers of approximately sevenfold. A promoter polymorphism linked to UGT2B7*2 has been suggested to decrease rates of transcription of the UGT2B7 enzyme [23]. However, the *in vitro* data in this study support *in vivo* data indicating no conclusive evidence of significant effect of the UGT2B7*2 polymorphisms on the pharmacokinetics of UGT2B7 substrates [7, 24–25]. Additionally, oral clearance of the UGT2B7 substrate gemcabene [15] was unimodal in a study of 300 individuals (Mark Milad, personal communication, Pfizer Global Research and Development).

Multiple factors contribute to variability in drug clearance for glucuronidated drugs [26], although there is no evidence for sex differences *in vivo*, consistent with the findings in this study. Mean clearance values are twofold lower in neonates compared with adults and are 1.4-fold lower in the elderly [26]. Also, mean clearances are twofold lower in patients with liver or renal disease compared with healthy individuals [25, 26]. These effects may be due to changes in liver blood flow (for high hepatic extraction drugs), or hepatic glucuronidation, or a combination of both. As stated above, co-administered drugs may also influence expression levels of the inducible UGT1A1 gene [25]. Thus, although the analysis described in this study demonstrates functional consequences of hepatic glucuronidation for the UGT1A1*28 polymorphism, this is likely only partially to explain the observed variability in hepatic clearance for drugs primarily glucuronidated by UGT1A1. It is also interesting to observe that clearances for drugs glucuronidated by UGT2B7 are observed to be more variable than drugs glucuronidated by other enzymes [26], which may be related to the blood flow limited clearance of high extraction UGT2B7 substrates such as AZT and morphine. Based on the findings of this study, the UGT2B7*2 polymorphism does not appear to alter expression levels of UGT2B7, or the rate of UGT2B7-catalysed AZTG. However, this does not preclude the possibility of altered binding affinity for other substrates for the enzyme encoded by genes with the UGT2B7*2 polymorphism.

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