A Humanized UGT1 Mouse Model Expressing the

UGT1A1*28 Allele for Assessing Drug Clearance by

UGT1A1- Dependent Glucuronidation

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Nonstandard abbreviations:

AUC = area under the curve, PEG = polyethylene glycol; PBS = phosphate buffered saline;
NMP = N-methylpyrrolidone; wt = C57BL/6 mouse; $Tg(UGT1A1^{*28})Ugt1^{-/-}$ = humanized $UGT1$ mice that express the $UGTIA1^{*28}$ allele, pb = phenobarbital.
ABSTRACT

Humanized mice that express the human *UDP-glucuronosyltransferase (UGT)* 1 locus have been developed in a *Ugt1*-null background as a model to improve predictions of human UGT1A dependent drug clearance. Enzyme kinetic parameters (K\text{m} and V\text{max}) and pharmacokinetic properties of three probe drugs were compared using wild-type and humanized *UGT1* mice that express the Gilbert’s UGT1A*28* allele (*Tg(UGT1A*28*) *Ugt1*\text{−/−} mice). The well characterized substrate for UGT1A1, 7-ethyl-10-hydroxy-camptothecin (SN-38), showed the greatest difference in parent drug exposure (~ 3-fold increase) and clearance (~ 3-fold decrease) in *Tg(UGT1A*28*) *Ugt1*\text{−/−} mice following intravenous administration when compared to wild type and phenobarbital-treated animals. In contrast, the clearance of the UGT2B7 substrate (-)-17-allyl-4, 5\text{α}-epoxy-3, 14-dihydroxymorphinan-6-one (naloxone) was not altered in *Tg(UGT1A*28*) *Ugt1*\text{−/−} mice. In addition, pharmacokinetic parameters with 1-(4-fluorophenyl)3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone (ezetimibe (Zetia)), considered to be a major substrate for UGT1A1, showed small to no dependence on UGT1A1 directed glucuronidation. Enzyme kinetic parameters assessed for SN-38, ezetimibe, and naloxone using liver microsomes prepared from wild-type and *Tg(UGT1A*28*) *Ugt1*\text{−/−} mice showed patterns consistent with the in vivo pharmacokinetic data. For SN-38 glucuronidation, V\text{max} decreased 5-fold in *Tg(UGT1A*28*) *Ugt1*\text{−/−} mouse liver microsomes when compared to microsomes prepared from wild-type mice and 10-fold when compared to phenobarbital-treated *Tg(UGT1A*28*) *Ugt1*\text{−/−} mice. These differences are consistent with SN-38 glucuronidation activities using human liver
microsomes isolated from individuals genotyped as UGT1A1*1 or UGT1A1*28. For ezetimibe and naloxone the differences in V\text{max} were minimal. Thus, Tg(UGT1A1*28) Ugt1\text{−/−} mice can serve as a pharmacokinetic model to further investigate the effects of UGT1A1 expression on drug metabolism.
Introduction

UDP-glucuronosyltransferases (UGTs) exist as an enzyme “superfamily” and are quantitatively among the most important drug metabolizing enzymes. Together with cytochrome P450, they are responsible for the elimination of more than 90% of hepatically cleared drugs (Miners et al., 2004). Within the UGT superfamily, the nine UDP-glucuronosyltransferases (UGTs) encoded by the UGT1 locus in humans are responsible for the conjugation of most exogenous compounds (such as drugs, environmental toxicants and carcinogens) and endogenous substances (such as bile acids, fatty acids, steroids, hormones, and bilirubin) (Tukey and Strassburg, 2000; Miners et al., 2002). They exhibit distinct, but often overlapping, substrate and inhibitor selectivity and differ in the occurrence and frequency of genetic polymorphism and regulation (Tukey and Strassburg, 2000; Miners et al., 2002). Of particular interest among the UGT1A subfamily is UGT1A1 (Bosma, 2003). This enzyme is solely responsible for the glucuronidation of bilirubin and contributes to the conjugation of exogenous and endogenous substances, including SN-38, the active metabolite of irinotecan (Iyer et al., 1998; Strassburg et al., 2000; Bosma, 2003).

Numerous polymorphisms in the UGT1A1 gene have been identified (Udomuksorn et al., 2007). A common variant with functional consequence is the UGT1A1*28 allele, resulting from a (TA) insertion into the UGT1A1 promoter region. The UGT1A1*28 genotype has been linked to Gilbert’s syndrome, and these individuals have been reported to be subject to episodes of mild hyperbilirubinemia. In addition, this population may have altered pharmacokinetic drug profiles and more importantly, are susceptible to the
dose/exposure-limited toxicities exemplified by the anticancer drug irinotecan (CPT-11) (Iyer et al., 2002; Tukey et al., 2002; Nagar and Blanchard, 2006). Even though the underlying mechanism for irinotecan toxicity is yet to be determined, it is linked to the decreased hepatic UGT1A1 activity observed in Gilbert’s syndrome, leading to reduced systemic clearance and consequently sustained elevated levels of SN-38, the active form of CPT-11 (Iyer et al., 1998; Iyer et al., 1999; Gagne et al., 2002). Human liver microsomes, isolated from individuals with the \textit{UGT1A1*28} polymorphism, demonstrate a decrease in catalytic activity toward SN-38 and other UGT1A1 substrates. Thus, genotyping for the \textit{UGT1A1*28} allele is recommended along with appropriate dose adjustments for CPT-11 treatment (O’Dwyer and Catalano, 2006; Ando et al., 2007).

Despite the fact that the \textit{UGT1A1*28} allele represents a common polymorphism and has been shown to lead to a clinically relevant phenotype, there are few tools to assess and predict whether lower UGT1A1 expression will affect the overall clearance of a new chemical entity. This is due in part to the lack of tools for UGT reaction phenotyping, such as specific chemical substrates, chemical and antibody inhibitors, and well-characterized expressed enzymes (Miners et al., 2009). Currently, the most widely used and effective tool for attributing substrate specificity to a specific UGT is expressed UGT enzymes (Ethell et al., 2001). However, the utility of an expressed enzyme can be complicated by several factors shown recently to alter the kinetics of glucuronidation. These include, but are not limited to, bovine serum albumin effects (Miners et al., 2006; Rowland et al., 2008), UGT binary complex formation (Fujiwara et al., 2007), co-expression of multiple UGTs (Fujiwara et al., 2007) and co-expression of UGTs with CYP3A4 (Takeda et al.,
2005; Ishii et al., 2007). Alternatively, a more laborious approach, with limited utility because of the lack of specific substrates for a given UGT isozyme, is the correlation analysis of an activity known to be specific for a single UGT with the glucuronidation of an unknown compound across an array of individual human liver microsomes (Court, 2005). However, caution should be taken when drawing any conclusions from these data without any confirmatory data obtained from other methods.

An alternative approach to assess the substrate potential of a new chemical entity for UGT1A1, and thus potentially assess the likelihood for altered pharmacokinetics in individuals with the UGT1A1*28 genotype, is the implementation of humanized mice. In this report, humanized mice that carry the UGT1A1*28 allele and have been shown to duplicate the hyperbilirubinemia condition present in Gilbert’s syndrome were evaluated for the first time as a model for assessing UGT1A1-related clearance and metabolism. Thus, three literature compounds were chosen for this research: SN-38 (an exclusive UGT1A1 substrate (Iyer et al., 1998)), ezetimibe (a partial UGT1A1 substrate (Ghosal et al., 2004)), and naloxone (a UGT2B7 substrate). Pharmacokinetic and enzyme kinetic parameters for SN-38, ezetimibe and naloxone in wild-type and humanized UGT1 mice were compared. The data is discussed in the context of evaluating the suitability of the humanized mouse model for assessing UGT1A1-related clearance and metabolism for humans.
Methods

Materials. Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Human liver microsomes were purchased from BD Gentest (BD Biosciences, Woburn, MA). SN-38 and ezetimibe were synthesized internally by Pfizer Global Research and Development laboratories. Naloxone was purchased from Sigma Aldrich (St Louis, MO). Phenobarbital was purchased from Henry Schein Inc. (Melville, NY).

Generation of humanized UGT1 mice. Transgenic mice that express the human UGT1 locus (Tg-UGT1) have been reported (Chen et al., 2005), and these studies have shown the expression of the UGT1A genes in humanized UGT1 mice are identical to those shown for expression of the UGT1 locus in Tg-UGT1 mice. In addition, these patterns closely resemble the expression patterns observed in human tissues. DNA sequence analysis of the UGT1A1 promoter that is expressed in Tg-UGT1 mice contains an extra (TA) insertion in the TATAA element (A(TA)7TAA) which identifies the gene as the human UGT1A1*28 allele. Breeding experiments with Tg-UGT1 mice and Ugt1+/- mice (Nguyen et al., 2008) led to a cross that expressed the UGT1 and Ugt1+/- alleles, that when backcrossed produced mice that were transgenic for the UGT1 locus in a Ugt1+/- background. Since the UGT1 locus encodes the nine UGT1A genes which includes the UGT1A1*28 allele, these mice were identified as being humanized for the UGT1A1*28 gene and are designated as Tg(UGT1A1*28)Ugt1+/- mice.

Microsomal Enzyme Kinetics. Liver microsomes from wild type (C57BL/6) and Tg(UGT1A1*28)Ugt1+/- mice were prepared as previously described (Chen et al., 2005).
Using either human liver or mouse liver microsomes, the following procedure was utilized. Incubations, in triplicate, containing 50 mM Tris (pH 7.4), 0.25 mg/mL microsomal protein, 5 mM MgCl₂, 50 µg/mL alamethicin, and 5 mM UDPGA in a final concentration of 200 µL, were conducted in 96 deep-well plates at 37°C for 30 min. The reactions were stopped by the addition 100 µL of cold 0.1% formic acid in acetonitrile containing 0.25 µM carbamazepine as the internal standard. The samples were briefly centrifuged and the supernatants were analyzed using LC/MS/MS. For substrate concentration-dependent glucuronidation activities of selected human liver microsomes (donor designations of HH9 and HH81, each genotyped as *28/*28; and donors HH83 and HH112, each genotyped as *1/*1) and mouse liver microsomes, the substrate concentrations were 0.49 to 100 µM for ezetimibe; 0.23 to 30 µM for SN-38; and 7.8 to 1000 µM for naloxone.

**LC/MS/MS Conditions and Data Analysis for Enzyme Kinetics.** No glucuronide standards were used for quantitation. Rather, quantitation was accomplished by using SN-38, ezetimibe, or naloxone as a standard, and assuming similar ionization between parent and glucuronide. Samples were analyzed by LC/MS/MS in positive mode utilizing a Thermo Aquasil C18 2.1×20 mm, 3 µm column at a flow rate of 0.5 mL/min (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile). Initial conditions were 5% B at 0.5 mL/min followed by a linear gradient from 5 to 95% B in 1 min, hold at 95% B for 1 min, and then a 1-min re-equilibration at 5% B. Mass transitions and retention times for the SN-38 glucuronide, ezetimibe glucuronide, naloxone glucuronide, and carbamazepine (IS) were 569→393 at 1.06 min, 586→410 at 1.24 min, 504→328 at 0.76
min and 237→194 at 1.29 min, respectively. Standard curves were fitted to a 1× weighted linear regression model and correlation coefficients were ≥ 0.99.

The $K_m$ and $V_{max}$ values of each enzyme were estimated by fitting the glucuronidation activities to the Michaelis-Menten equation using a nonlinear regression analysis in GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA).

**Pharmacokinetic studies in mice.** Wild-type and Tg(UGT1A1*28)Ugt1−/− mice were single or double housed in polycarbonate microisolator cages with contact bedding upon arrival. Animal room temperature and humidity were targeted to be between 70±5°F and 30-70%, respectively. Lighting was set to maintain an approximate 12 hour light, 12 hour dark cycle. Food (Purina Certified Rodent Diet 5002) and water were available *ad libitum*. Animal housing and husbandry were performed in accordance with the provisions of the *Guide for the Care and Use of Laboratory Animals*. The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

The mice were weighed the morning of dosing and the dose volumes were calculated based on body weights (Table 1). Intravenous dose volumes for the mouse studies were either 4 mL/kg or 8 mL/kg. Blood was collected into lithium heparin blood tubes at various time points via retro-orbital (RO) bleeding under general anesthesia (80% CO$_2$/20%O$_2$). A terminal blood sample was taken from the posterior vena cava following euthanasia using CO$_2$. The blood was separated into plasma by centrifugation and then aliquotted and stored at -80°C until analyzed.
In one study, male humanized mice were dosed via intraperitoneal (IP) injection for 4 days with 100 mg/kg/day sodium phenobarbital (12 mg/mL free base equivalent; 8 mL/kg) in sterile water. On the fifth day the mice received SN-38 via IV administration and blood was collected at various time points as previously described (n=2 animals per time point for each study). After the terminal bleed (last time point), livers were removed, rinsed in saline, blotted dry and frozen on dry ice. Livers were stored at -80°C until microsome preparation and Western blot analysis.

Sample Preparation, LC/MS/MS Conditions and Data Analysis for Mouse Pharmacokinetic Studies. Mouse plasma samples were prepared using a protein precipitation procedure with acetonitrile containing analytical internal standard (carbamazepine) and analyzed utilizing an LC/MS/MS system comprised of a SCL-10A VP Shimadzu pump LC system controller (Columbia, MD), coupled with a triple quadrupole mass spectrometer using multiple reaction monitoring in the positive electrospary ionization mode (API4000 SCIEX, Sunnyvale, CA). The separation of the analytes was achieved by using an Aquasil C18 20×2.1mm (3µm) (Thermo Scientific, Waltham, MA) column. A 3.5 min gradient at 0.5 mL/min flow rate was applied with the following mobile phases: A: 0.1% formic acid in water, B: acetonitrile with 0.1% formic acid. The same linear elution LC gradient was used as described for the in vitro enzyme kinetic experiments. Mass transitions and retention times for SN-38, ezetimibe, naloxone, and internal standard (carbamazepine) were 393.0→249.2 at 1.08 min, 410.1→132.9 at 1.4 min, 328.25→253.1 at 0.95 min, and 237.0→194.0 at 1.28 min respectively. Quantitation
of the analytes was performed using Analyst 1.2 software (Applied Biosystems, Carlsbad, CA).

Pharmacokinetic calculations were performed using the non-compartmental approach with the aid of Watson LIMS (Version 7.2.0.03, Thermo Electron Corp). The initial plasma concentration \( (C_0) \) was determined by linear regression extrapolation from the first 2 data points from mean animals. The area under the plasma concentration-time curve \([\text{AUC}(0-t)]\) was determined using the linear trapezoidal method.

**Immunoblot Analysis of Humanized UGT1 Mouse and Human Liver Microsomes.** All Western blots were performed using NuPAGE BisTris-polyacrylamide gels as outlined by the supplier (Invitrogen, Carlsbad, CA). Protein was heated at 70°C for 10 min in loading buffer and resolved in 4–12% Bis-Tris gels under denaturing conditions (50 mM MOPS, 50 mM Tris-base, pH 7.7, 0.1% SDS, 1 mM EDTA) prior to transferring the proteins to a polyvinylidene difluoride membrane using a semidry transfer system (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20 (Tris-buffered saline) for 1 h at room temperature, followed by incubation with primary antibodies (mouse anti-human UGT1A1, a gift from Prof. Joseph K. Ritter, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA) in Tris-buffered saline overnight at 4°C. Membranes were washed and exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Each membrane was washed again, and the conjugated horseradish peroxidase was detected using the ECL plus Western
blotting detection system (Amersham Biosciences, Sweden), and the proteins were detected following exposure to X-ray film.

**RNA preparation and RT-PCR analysis.** Pooled liver tissues were pulverized in liquid nitrogen, approximately 100 mg of tissue was homogenized into 1 mL of TRIzol (Invitrogen, Carlsbad, CA). Two hundred μL of chloroform was added with vigorously shaking. The solution was centrifuged, and the aqueous phase was transferred to a new tube. The RNA was precipitated down by adding 500 μL of isopropanol, collected by centrifugation, and followed by 75% ethyl alcohol wash. The RNA was dissolved into DEPC water and the concentration was determined. Using Iscript Reverse Transcriptase (Bio-Rad, Hercules, CA), 1 μg of total RNA in a volume of 20 μL was used for the generation of cDNA as outlined by the manufacturer. Thereafter, synthesized cDNA was used for amplification of human UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9, as described previously (Chen et al., 2005). In brief, the PCR reaction with 1 μL cDNA and 0.25 μM of each of the UGT1A-specific oligonucleotide primer pair was carried out in a total volume of 20 μL by polymerase master mix (Denville Scientific, South Plainfield, NJ). The polymerase was activated at 95 °C for 10 minutes followed by 30 cycles of 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. Following the reaction, 15 μL of the PCR products were subjected to electrophoresis in 1.5 % agarose gels and documented by ChemiDoc XRS gel imaging system (Bio-Rad, Hercules, CA).
Results

**Mouse Pharmacokinetic Studies.** Pharmacokinetic parameters were determined in mice (intravenous route) for three established UGT substrates: SN-38, the active metabolite of irinotecan which is cleared predominantly by UGT1A1, ezetimibe (Zetia) which is believed to form the phenolic glucuronide by UGT1A1, 1A3, and 2B15 (ezetimibe also forms a very minor benzylic glucuronide by UGT2B7 which is not monitored in this report), and naloxone which is a UGT2B7 substrate (Figure 1). In the case of SN-38, at the doses tested, no adverse effect was seen with either wild-type C57BL/6 or Tg(UGT1A*28)Ugt1-/− mice. The pharmacokinetic results are summarized in Table 2 and displayed in Figure 2.

**Wild-type C57BL/6 mouse studies.** All three compounds were rapidly cleared in wild-type mice at the dose levels evaluated. For the 1 mg/kg intravenous (IV) dose, SN-38 exhibited a clearance of 320 mL/min/kg and an Area Under the Curve (AUC) of 0.14 μM·h, whereas at the 2 mg/kg IV dose the clearance was lower and the exposure was higher (clearance of 230 mL/min/kg and an AUC of 0.37 μM·h, respectively). For ezetimibe, 1 mg/kg IV yielded a clearance value of 136 mL/min/kg and an AUC 0.30 μM·h. At higher doses (5 mg/kg), clearance was constant while the AUC increased approximately 5-fold. Naloxone, at 5 mg/kg IV, had a high clearance of 194 mL/min/kg and an AUC of 1.3 μM·h.

**Humanized UGT1 mouse studies.** The development of Tg(UGT1A*28)Ugt1-/− mice leads to minimal expression of hepatic UGT1A1 (Figure 3). In 8 week old mice, the serum bilirubin levels are mildly elevated when compared to same age wild-type mice, a
finding that links the TATAA promoter polymorphism in the UGT1A1*28 gene to the observed hyperbilirubinemia. Thus, drugs that enter the circulation and become targeted for UGT1A1-dependent glucuronidation would be expected to show differences in clearance and AUC. For SN-38, the AUC increased from 0.54 to 0.87 μM·h as the dose increased from 1 to 2 mg/kg, whereas clearance was fairly consistent (80 versus 96 mL/min/kg). When the mice were treated with phenobarbital prior to administration of SN-38, hepatic UGT1A1 expression was greatly induced (Figure 3). In addition, at the gene level, all the UGT1A genes expressed in human liver (except for UGT1A6 which was only mildly induced) were significantly induced in these mice following phenobarbital treatment (Figure 4). Consistent with the induction of UGT1A1, clearance of SN-38 in phenobarbital-treated mice increased (96 to 285 mL/min/kg), an observation that is concordant with a reduction in AUC (0.87 to 0.15 μM·h).

When we examined the pharmacokinetic parameters of ezetimibe at a 1 mg/kg dose, there was very little difference in the pharmacokinetic parameters when comparing Tg(UGT1A1*28)Ugt1−/− mice to wild-type mice. At a higher dose of ezetimibe (5 mg/kg), small differences (approximately 2-fold) in pharmacokinetic parameters were seen between Tg(UGT1A1*28)Ugt1−/− and wild-type mice. Finally, naloxone pharmacokinetics were evaluated, the AUC and clearance values were nearly identical when comparing results between wild-type and Tg(UGT1A1*28)Ugt1−/− mice.

**Enzyme kinetics.** The kinetics of glucuronidation were evaluated for the three compounds by monitoring the formation of glucuronide metabolites using liver microsomes. Each compound was investigated using either human liver microsomes...
(HLM) genotyped for the normal (UGT1A1*1) and the Gilbert’s (UGT1A1*28) allele, in addition to mouse liver microsomes (MLM) prepared from untreated wild-type mice, Tg(UGT1A1*28)Ugt1<sup>−/−</sup> mice, and phenobarbital-treated Tg(UGT1A1*28)Ugt1<sup>−/−</sup> mice. In all cases, parent (not glucuronide) standards were used for quantitation. The enzyme kinetic parameters with HLMs and the various MLMs are reported in Table 3. Individual data and data fitting are displayed in Figure 5 (for HLM) and Figure 6 (for MLM).

**Enzyme kinetics with HLM.** Four individual lots of HLM were obtained commercially from BD Gentest (Woburn, MA). They were genotyped in house and Western blot analysis was also performed (Figure 3) to ensure relative UGT1A1 protein content in individual HLM was consistent with genotype (Fang and Lazarus, 2004; Peterkin et al., 2007). This figure clearly shows a dramatic reduction of UGT1A1 protein in each of the microsomal preparations of UGT1A1*28 origin. For the kinetic analysis with SN-38, the K<sub>m</sub> ranged from 0.635 to 1.46 μM whereas V<sub>max</sub> decreased 4- to 8-fold between HLM genotyped for the UGT1A1*1 and UGT1A1*28 alleles (Table 3).

For ezetimibe, K<sub>m</sub> ranged from 5.18 to 16.9 μM and V<sub>max</sub> from 781 to 1619 pmol/min·mg with UGT1A1*1 and UGT1A1*28 HLM, respectively. In the case of naloxone, the observed differences in K<sub>m</sub> (ranging from 34.5 to 63.1 μM) or V<sub>max</sub> (ranging from 1593 to 2059 pmol/min·mg) were minimal, as expected.

**Enzyme kinetics with MLM.** The enzyme kinetic results using MLMs prepared from wild-type and Tg(UGT1A1*28)Ugt1<sup>−/−</sup> mice in addition to Tg(UGT1A1*28)Ugt1<sup>−/−</sup> mice treated with phenobarbital are provided in Table 3. For SN-38, the K<sub>m</sub> values ranged from 1.44 μM in MLM prepared from phenobarbital-treated Tg(UGT1A1*28)Ugt1<sup>−/−</sup> mice to 3.59
μM in wild-type MLM. The V_{\text{max}} increased from 43.6 pmol/min·mg in untreated Tg(UGT1{A1}^{*28})Ugt1^{-/-} mice to 497 pmol/min·mg in MLM prepared from phenobarbital-treated Tg(UGT1{A1}^{*28})Ugt1^{-/-} mice (~11-fold), a finding which supports the robust induction of UGT1A1 in microsomes from phenobarbital-treated Tg(UGT1{A1}^{*28})Ugt1^{-/-} mice. When ezetimibe and naloxone were evaluated, there were no differences in K_{m} and V_{\text{max}} values when using microsomes from untreated and phenobarbital-treated Tg(UGT1{A1}^{*28})Ugt1^{-/-} mice.
Discussion

Given the importance of cytochrome P450 and UDP-glucuronosyltransferases in the metabolism and elimination of exogenous and endogenous xenobiotics, there is continuous interest in developing techniques and tools to identify the P450 and UGT enzyme(s) involved in the metabolism of a new drug and, additionally, in predicting human pharmacokinetic parameters to either guide compound selection in a discovery mode or select clinical doses in a development setting using in vitro kinetic parameters or in vivo preclinical pharmacokinetic data (Beaumont and Smith, 2009). Although qualitative and quantitative in vitro-in vivo correlation analysis based on data generated using human liver tissues and recombinant enzymes has been applied successfully to many drugs that are cleared by P450-dependent metabolism, these approaches have proven less definitive for glucuronidated compounds mainly for the reasons alluded to earlier (Soars et al., 2003; Miners et al., 2004; Kilford et al., 2009). Additionally, allometric scaling based on in vivo preclinical models has been successful for P450-eliminated drugs, provided that species specific P450 enzymes have been well studied and understood (Caldwell et al., 2004; De Buck et al., 2007). Conversely, given the current challenge of using preclinical species to predict human pharmacokinetics for predominantly glucuronidated compounds, more research is required to fully understand species differences and substrate specificity of UGT enzymes. Clearly, genetically modified mouse models, in which a specific gene is removed or replaced, have proven to be useful in studying drug metabolizing enzymes (namely P450 enzymes) and drug transporters even though quantitative prediction of the kinetic parameters of drugs is often limited by the complexity of gene transcription and
translation and other factors, such as differences in levels and tissue expression patterns of enzymes in genetically modified mouse models (Lin, 2008). The recent generation of Tg(UGT1A1*28)Ugt1-/- mice has the potential to address issues of UGT-dependent drug clearance and toxicity, since the humanization process does not alter expression of the constitutive mouse specific UGT2 enzymes, as we have demonstrated by Western blot analysis using UGT2B specific antibodies (Nguyen et al., 2008). These humanized mice have been shown to be mildly hyperbilirubinemic, which serves as a biomarker of, and is fully consistent with, the expression of UGT1A1*28 and subsequent decrease in UGT1A1 specific glucuronidation (decreased V_max).

To validate the utility of the humanized mouse model for studying hepatic glucuronidation, three literature compounds were chosen to cover a range of UGT1A1 involvement in the elimination of these compounds. The compounds evaluated were, SN-38, the active metabolite of irinotecan which is cleared predominantly by UGT1A1, ezetimibe (Zetia) which is reported to be eliminated partially by UGT1A1, and naloxone which is not a UGT1A1 substrate (Figure 1). Irinotecan and SN-38 have been well characterized, due to their adverse effect (diarrhea) in some patients (Iyer et al., 1998; Iyer et al., 1999; Onoue and Inui, 2008). Following intravenous administration of a 1 mg/kg dose in mice, SN-38 showed approximately 4-fold differences in parent drug exposure (AUC) and clearance in the humanized mice when compared to wild type mice. The extremely high clearance values for SN-38 were likely due to the extrahepatic elimination reported for the compound in rats (Chu et al., 1997). Similar differences in AUC and clearance were also seen between humanized and phenobarbital-treated
Tg(UGT1A1*28)Ugt1−/− mice. Since UGT1A1 is efficiently induced by phenobarbital in Tg(UGT1A1*28)Ugt1−/− mice and others have shown that SN-38 is glucuronidated primarily by UGT1A1, these results are consistent with SN-38 glucuronidation by UGT1A1. In enzyme kinetic studies with HLM genotyped for normal UGT1A1*1 and variant UGT1A1*28, V_{max} of SN-38 glucuronidation decreased 4- to 8-fold when comparing the *1/*1 and *28/*28 samples. These results are consistent with differences in protein concentration, with results reported by another laboratory where V_{max} values showed a >10-fold difference using HLMs that were genotyped for the two UGT1A1 allelic variants (Zhang et al., 2007), and with the in vivo differences in pharmacokinetic parameters between phenobarbital-treated and untreated Tg(UGT1A1*28)Ugt1−/− mice. Additionally, they follow the same rationale that the expression of UGT1A1 in liver microsomes obtained from UGT1A1*28 individuals would only affect the catalytic activity (with decreased catalytic activity, V_{max}) but not the substrate affinity (K_{m}) given that the only difference is reduced liver expression of UGT1A1 (Iyer et al., 1998; Gagne et al., 2002). Furthermore, the change in V_{max} between HLMs genotyped as UGT1A1*1 compared with UGT1A1*28 supports the clinical finding that Gilbert’s patients are more prone to adverse events with irinotecan therapy (Iyer et al., 2002; Tukey et al., 2002). With MLM prepared from wild type and Tg(UGT1A1*28)Ugt1−/− mice (with and without phenobarbital treatment) the V_{max} for SN-38 glucuronidation demonstrated similar trends to those determined in HLM. Specifically, the increases in V_{max} from humanized MLM to wild-type MLM and to phenobarbital-treated Tg(UGT1A1*28)Ugt1−/− microsomes were approximately 5- and 11-
fold, respectively, which implies a direct translation of in vitro enzyme kinetic parameters from MLM to HLM.

In contrast to SN-38, the clearance of the UGT2B7 substrate, naloxone (Coffman et al., 1998), was not altered in the humanized mice when compared to wild-type mice, given that it is not a UGT1A1 substrate. Consistent with this in vivo observation, any differences in the maximal velocity of naloxone glucuronidation were minimal between HLMs genotyped for normal UGT1A1*1 and UGT1A1*28, or between MLMs prepared from Tg(UGT1A1*28)Ugt1−/− mice with or without phenobarbital treatment.

Using recombinant UGT preparations, Ghosal et al previously showed ezetimibe to be a substrate for multiple human UGT forms (UGT1A1, UGT1A3, UGT2B7 and UGT2B15 (Ghosal et al., 2004)). Our investigation began with mouse pharmacokinetic determinations, and with ezetimibe administration to wild-type and Tg(UGT1A1*28)Ugt1−/− mice at 1 mg/kg IV, clearance of the drug was found to be very similar in both strains. At a higher dose (5 mg/kg), only a 2-fold increase in AUC and a 2-fold decrease in clearance were seen, which is likely due to enzyme (UGT1A1) saturation in Tg(UGT1A1*28)Ugt1−/− mice (given the low protein levels (Figure 3)). Overall, this was a surprising observation considering previous reports that ezetimibe served as a major substrate for recombinant UGT1A1. In addition, the expression of UGT1A1 in Tg(UGT1A1*28)Ugt1−/− hepatic tissue is minimal, as shown by Western blot analysis. To further evaluate this observation, ezetimibe glucuronidation was examined in microsomes from phenobarbital-treated Tg(UGT1A1*28)Ugt1−/− mice, where induction of human UGT1A1 was robust. The K_m and V_max values as determined in MLM from untreated and phenobarbital-treated mice were
virtually identical, which is consistent with what was seen with HLM expressing either normal \textit{UGT1A1}*1 or \textit{UGT1A1}*28. Importantly, the rate of ezetimibe glucuronide formation was not reduced in HLM samples genotyped as \textit{UGT1A1}*28 when compared to normal \textit{UGT1A1}*1 HLMs, indicating that glucuronidation by UGT1A1 may not play a major role in overall ezetimibe metabolism. Since phenobarbital leads to the induction of UGT1A4 (Argikar et al., 2009) and we have observed significant induction of UGT1A3 and UGT1A9 and mild induction of UGT1A6 in Tg-\textit{UGT1} mice (Figure 4), it is instead likely that ezetimibe conjugation is catalyzed by UGT2B proteins. These results also indicate that the interpretation of enzymatic efficiency and substrate specificity using only expressed forms of the human UGTs must be interpreted with caution. The UGT functional specificity as they operate in vivo, which may be dependent upon tissue specific factors in addition to variations in heterodimer formation (Operana and Tukey, 2007), may result in glucuronidation efficiencies that are much different than those observed in vitro using expressed forms of single UGTs.

In summary, the \textit{Tg(UGT1A1*28)}\textit{Ugt1}⁻ mice have proven to be a useful tool to study UGT1A1 related drug clearance in vivo and in vitro and have the ability to at least qualitatively predict UGT1A1-involved clearance. Human liver UGT1A1 expression in individuals homozygous for the \textit{UGT1A1}*1 allele can result in up to 10-fold greater protein concentration in liver compared to those that are homozygous for \textit{UGT1A1}*28. These differences reflect similar patterns of human UGT1A1 expression in humanized mice when comparing their levels in untreated and phenobarbital-treated mice. These similarities validate the use of humanized \textit{UGT1} mice to examine pharmacokinetic
parameters in vivo especially when substrates are selectively metabolized by UGT1A1. Recent experiments using transgenic UGT1 mice have been employed successfully to examine the pharmacokinetic parameters of lamotrigine, a popular anti-epileptic drug that is metabolized by human UGT1A4 (Argikar et al., 2009). With current human safety assessments of new drugs relying primarily on standard animal models and in vitro experiments, coupled with arguments that intrinsic clearance values that rely on in vitro microsomal metabolism studies under-predict in vivo clearance values by an order of magnitude (Miners et al., 2006), the introduction of humanized animals modeled to express drug and xenobiotic metabolizing enzymes should be perceived as a valued addition (Cheung and Gonzalez, 2008) towards providing accurate information in predicting human drug metabolism.
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References


Peterkin VC, Bauman JN, Goosen TC, Menning L, Man MZ, Paulauskis JD, Williams JA and Myrand SP (2007) Limited influence of UGT1A1*28 and no effect of
UGT2B7*2 polymorphisms on UGT1A1 or UGT2B7 activities and protein expression in human liver microsomes. *Br J Clin Pharmacol* **64**:458-468.


Footnotes

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Legends for figures

Figure 1. Glucuronidation reactions investigated in this study.

Figure 2. Time courses of SN-38, ezetimibe and naloxone in mice dosed intravenously (n of 2 mice per time point and each data point shown represents the average of 2 determinations). (diamonds = wild type C57BL/6 mice, squares = humanized \( \text{UGT1} \) mice, and triangles = humanized \( \text{UGT1} \) mice with phenobarbital treatment).

Figure 3. Protein expression of \( \text{UGT1A1} \) in \( Tg(\text{UGT1A}^{\text{Al}^*28})\text{Ugt1}^{\text{I}^{-/}} \) male mouse liver and human liver microsomes. Mice were treated with phenobarbital at 100 mg/kg/day for 4 days. Left panel, control = \( Tg(\text{UGT1A}^{\text{Al}^*28})\text{Ugt1}^{\text{I}^{-/}} \) mice without phenobarbital treatment; pb = \( Tg(\text{UGT1A}^{\text{Al}^*28})\text{Ugt1}^{\text{I}^{-/}} \) mice with phenobarbital treatment. Human liver microsomes have been genotyped for expression of the \( \text{UGT1A1}^{*28} \) or \( \text{UGT1A1}^{*1} \) alleles.

Figure 4. Human UGT1A gene expression in \( Tg(\text{UGT1A}^{\text{Al}^*28})\text{Ugt1}^{\text{I}^{-/}} \) mice treated with phenobarbital. Six age-matched humanized \( Tg(\text{UGT1A}^{\text{Al}^*28})\text{Ugt1}^{\text{I}^{-/}} \) mice were treated intraperitoneally with either DMSO (control) or phenobarbital (100 mg/kg) for 4 consecutive days. The day after the last dose the mice were sacrificed, and liver tissues were collected and pooled (n=3). Liver total RNA was prepared, followed by RT-PCR to determine the expression levels of human UGT1A1, UGT1A3, UGTA4, UGT1A6, and UGT1A9.
Figure 5. Substrate concentration-dependent glucuronidation of SN-38 (A), ezetimibe (B), and naloxone (C) by HLM genotyped as *UGT1A1*/*28/*28 (HH9 and HH81) and *UGT1A1*/*1/*1 (HH83 and HH112) (n of 3 replicates per data points). Open circles = HH83; open triangles = HH112; open squares = HH9; open diamonds = HH81.

Figure 6. Substrate concentration-dependent glucuronidation of SN-38 (A), ezetimibe (B), and naloxone (C) by mouse liver microsomes prepared from wt C57BL/6 mice (open circles), *Tg(UGT1A1*/*28)*Ugt1/− mice without phenobarbital treatment (open squares) and with phenobarbital treatment (open triangles) (n of 3 replicates per data points using pooled mouse liver microsomes prepared from a minimum of 3 animals).
Table 1. Intravenous formulation and time points for pharmacokinetic studies of SN-38, ezetimibe, and naloxone in wild-type and Tg(UGT1<sup>Atv28</sup>)Ugt1<sup>−/−</sup> mice (n = 2 animals per time point).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mouse type</th>
<th>IV dose (mg/kg)</th>
<th>Formulation</th>
<th>Time point range</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN-38</td>
<td>WT</td>
<td>1</td>
<td>10% ethanol/70% PEG-400/20% PBS</td>
<td>5 min – 8 hrs</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>2</td>
<td>10% ethanol/70% PEG-400/20% PBS</td>
<td>5 min – 3 hrs</td>
</tr>
<tr>
<td></td>
<td>Tg(UGT1&lt;sup&gt;Atv28&lt;/sup&gt;)Ugt1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1</td>
<td>10% ethanol/70% PEG-400/20% PBS</td>
<td>5 – 45 min</td>
</tr>
<tr>
<td></td>
<td>Tg(UGT1&lt;sup&gt;Atv28&lt;/sup&gt;)Ugt1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2</td>
<td>10% ethanol/70% PEG-400/20% PBS</td>
<td>2.5 min – 3 hrs</td>
</tr>
<tr>
<td></td>
<td>Tg(UGT1&lt;sup&gt;Atv28&lt;/sup&gt;)Ugt1&lt;sup&gt;−/−&lt;/sup&gt; w/ pb treatment</td>
<td>1</td>
<td>10% ethanol/70% PEG-400/20% water</td>
<td>3 – 80 min</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>WT</td>
<td>1</td>
<td>10% ethanol/50% PEG-400/40% PBS</td>
<td>5 min – 8 hrs</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>5</td>
<td>10% ethanol/50% PEG-400/40% PBS</td>
<td>5 min – 3 hrs</td>
</tr>
<tr>
<td></td>
<td>Tg(UGT1&lt;sup&gt;Atv28&lt;/sup&gt;)Ugt1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1</td>
<td>10% ethanol/50% PEG-400/40% PBS</td>
<td>5 – 45 min</td>
</tr>
<tr>
<td></td>
<td>Tg(UGT1&lt;sup&gt;Atv28&lt;/sup&gt;)Ugt1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5</td>
<td>10% ethanol/50% PEG-400/40% PBS</td>
<td>2.5 min – 3 hrs</td>
</tr>
<tr>
<td>Naloxone</td>
<td>WT</td>
<td>5</td>
<td>10% NMP/50% PEG-400/40% PBS</td>
<td>5 min – 8 hrs</td>
</tr>
<tr>
<td></td>
<td>Tg(UGT1&lt;sup&gt;Atv28&lt;/sup&gt;)Ugt1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5</td>
<td>10% NMP/50% PEG-400/40% PBS</td>
<td>5 min – 8 hrs</td>
</tr>
</tbody>
</table>
Table 2. Pharmacokinetic parameters of SN-38, ezetimibe, and naloxone in wild-type C57BL/6 and Tg(UGT1A1*28)Ugt1/ mice (dosed intravenously, n of 2 mice per time points, values in parentheses represent individual determinations).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mouse type</th>
<th>IV dose (mg/kg)</th>
<th>AUC (μM·h)</th>
<th>CL (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN-38</td>
<td>Wt</td>
<td>1</td>
<td>0.14 (0.15, 0.12)</td>
<td>320 (276, 363)</td>
</tr>
<tr>
<td></td>
<td>Wt</td>
<td>2</td>
<td>0.37 (0.34, 0.39)</td>
<td>230 (241, 219)</td>
</tr>
<tr>
<td></td>
<td>Humanized</td>
<td>1</td>
<td>0.54 (0.57, 0.50)</td>
<td>80 (74, 86)</td>
</tr>
<tr>
<td></td>
<td>Humanized</td>
<td>2</td>
<td>0.87 (0.79, 0.95)</td>
<td>96 (100, 92)</td>
</tr>
<tr>
<td></td>
<td>humanized w/ pb treatment</td>
<td>1</td>
<td>0.15 (0.18, 0.11)</td>
<td>285 (240, 330)</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>Wt</td>
<td>1</td>
<td>0.30 (0.31, 0.29)</td>
<td>136 (131, 141)</td>
</tr>
<tr>
<td></td>
<td>Wt</td>
<td>5</td>
<td>1.7 (1.67, 1.74)</td>
<td>120 (122, 117)</td>
</tr>
<tr>
<td></td>
<td>Humanized</td>
<td>1</td>
<td>0.31 (0.28, 0.35)</td>
<td>132 (145, 118)</td>
</tr>
<tr>
<td></td>
<td>Humanized</td>
<td>5</td>
<td>3.3 (3.60, 3.09)</td>
<td>63 (59, 66)</td>
</tr>
<tr>
<td>Naloxone</td>
<td>Wt</td>
<td>5</td>
<td>1.3 (1.2, 1.4)</td>
<td>194 (197, 191)</td>
</tr>
<tr>
<td></td>
<td>Humanized</td>
<td>5</td>
<td>1.2 (1.3, 1.1)</td>
<td>210 (199, 221)</td>
</tr>
</tbody>
</table>
Table 3. Kinetic parameters of glucuronidation from incubations with individual human and pooled mouse liver microsomes (minimal n of 3 animals and triplicate determinations per data point). Units were micromolar for $K_m$ values and pmol/min·mg for $V_{max}$ values.

<table>
<thead>
<tr>
<th>Liver Microsomes</th>
<th>SN-38</th>
<th>Ezetimibe</th>
<th>Naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH83 (wt)</td>
<td>0.964±0.274</td>
<td>162±10.9</td>
<td>5.18±2.28</td>
</tr>
<tr>
<td>HH112 (wt)</td>
<td>0.680±0.217</td>
<td>88.2±6.73</td>
<td>14.7±4.43</td>
</tr>
<tr>
<td>HH9 (*28/*28)</td>
<td>1.46±0.637</td>
<td>21.0±2.37</td>
<td>16.9±3.62</td>
</tr>
<tr>
<td>HH81 (*28/*28)</td>
<td>0.635±0.253</td>
<td>17.6±1.51</td>
<td>11.5±3.47</td>
</tr>
<tr>
<td>Mouse liver microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt C57BL/6 mouse</td>
<td>3.59±1.21</td>
<td>201±21.5</td>
<td>15.0±3.11</td>
</tr>
<tr>
<td>Tg(UGT1*28)Ugt1−/−</td>
<td>2.42±1.23</td>
<td>43.6±6.36</td>
<td>35.6±2.33</td>
</tr>
<tr>
<td>Tg(UGT1*28)Ugt1−/− with pb treatment</td>
<td>1.44±0.359</td>
<td>497±28.8</td>
<td>20.0±3.31</td>
</tr>
</tbody>
</table>
Figure 1

SN-38 → UGT1A1 → SN-38 glucuronide

Ezetimibe → UGT1A1 → Ezetimibe glucuronide

Naloxone → UGT2B7 → Naloxone glucuronide
Figure 2

- SN-38 at 1 mpk
- SN-38 at 2 mpk
- Ezetimibe at 1 mpk
- Ezetimibe at 5 mpk
- Naloxone at 5 mpk

Plasma Conc (μM) vs. Time (hour)
Figure 3

hUGT1*28 Liver control pb

Human Liver

*1/*1 *28/*28

51 — UGT1A1

UGT1A1
Figure 4

<table>
<thead>
<tr>
<th></th>
<th>UGT1A1</th>
<th>UGT1A3</th>
<th>UGT1A4</th>
<th>UGT1A6</th>
<th>UGT1A9</th>
</tr>
</thead>
<tbody>
<tr>
<td>pb</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

[Image of gel electrophoresis with bands for UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and pb]
Figure 5

A. SN-38 glucuronide (pmol/min/mg) vs. [SN-38] μM

B. Ezetimibe glucuronide (pmol/min/mg) vs. [Ezetimibe] μM

C. Naloxone glucuronide (pmol/min/mg) vs. [Naloxone] μM
Figure 6

A. SN-38 glucuronide (pmol/min/mg) vs. [SN-38] μM

B. Ezetimibe glucuronide (pmol/min/mg) vs. [Ezetimibe] μM

C. Naloxone glucuronide (pmol/min/mg) vs. [Naloxone] μM