A Humanized *UGT1* Mouse Model Expressing the *UGT1A1**28 Allele for Assessing Drug Clearance by *UGT1A1*-Dependent Glucuronidation

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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_m and V_{max}) and pharmacokinetic properties of three probe drugs were compared using wild-type and humanized *UGT1* mice that express the Gilbert's *UGT1A1**28 allele [*Tg(UGT1A1**28) *Ugt1*(-/-) 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(UGT1A1**28) *Ugt1*(-/-) mice after intravenous administration compared with wild-type and phenobarbital-treated animals. In contrast, the clearance of the *UGT2B7* substrate (-)-17-allyl-4, 5 α -epoxy-3, 14-dihydroxymorphinan-6-one (naloxone) was not altered in *Tg(UGT1A1**28) *Ugt1*(-/-) 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; Merck & Co., Whitehouse Station, NJ), 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(UGT1A1**28) *Ugt1*(-/-) mice showed patterns consistent with the in vivo pharmacokinetic data. For SN-38 glucuronidation, V_{max} decreased 5-fold in *Tg(UGT1A1**28) *Ugt1*(-/-) mouse liver microsomes compared with microsomes prepared from wild-type mice, and decreased 10-fold compared with phenobarbital-treated *Tg(UGT1A1**28) *Ugt1*(-/-) mice. These differences are consistent with SN-38 glucuronidation activities using HLMs isolated from individuals genotyped as *UGT1A1**1 or *UGT1A1**28. For ezetimibe and naloxone the differences in V_{max} were minimal. Thus, *Tg(UGT1A1**28) *Ugt1*(-/-) mice can serve as a pharmacokinetic model to further investigate the effects of *UGT1A1* expression on drug metabolism.

UDP-glucuronosyltransferases (UGTs) exist as an enzyme "superfamily" and are quantitatively among the most important drug-metabolizing enzymes. Together with cytochrome P450 (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 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 (CPT-11) (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 experience 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 CPT-11 (Iyer et al.,

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; SN-38, 7-ethyl-10-hydroxy-camptothecin; CPT-11, irinotecan; HLM, human liver microsome; MLM, mouse liver microsome; LC/MS/MS, liquid chromatography/tandem mass spectrometry; AUC, area under the curve.

2002; Tukey et al., 2002; Nagar and Blanchard, 2006). Even though the underlying mechanism for CPT-11 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, 1999; Gagné et al., 2002). Human liver microsomes (HLMs), isolated from individuals with the *UGT1A1**28 polymorphism, show a decrease in catalytic activity toward SN-38 and other UGT1A1 substrates. Thus, genotyping for the *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 *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 in part because of 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., 2010). 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), coexpression of multiple UGTs (Fujiwara et al., 2007), and coexpression 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 HLMs (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 are discussed in the context of evaluating the suitability of the humanized mouse model for assessing UGT1A1-related clearance and metabolism for humans.

Materials and Methods

Materials. Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). HLMs were purchased from BD Gentest (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 (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)₇ insertion in the TATAA element [A(TA)₇TAA] that 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, which when backcrossed produced mice that were transgenic for the *UGT1* locus in a *Ugt1*(-/-) background. Because the *UGT1* locus encodes the nine *UGT1A* genes, including 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 described previously (Chen et al., 2005). Using either HLMs or mouse liver microsomes (MLMs), the following procedure was used. 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 UDP-glucuronic acid 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 liquid chromatography/tandem mass spectrometry (LC/MS/MS). For substrate concentration-dependent glucuronidation activities of selected HLMs (donor designations of HH9 and HH81, each genotyped as *28/*28; and donors HH83 and HH112, each genotyped as *1/*1) and MLMs, 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 using a Thermo Fisher Scientific (Waltham, MA) 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 (internal standard) 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 Software Inc. (San Diego, CA) Prism 5.00 for Windows (Microsoft, Redmond, WA).

Pharmacokinetic Studies in Mice. Wild-type and Tg(*UGT1A1**28)*Ugt1*(-/-) mice were single- or double-housed in polycarbonate microisolator cages with contact bedding on arrival. Animal room temperature and humidity were targeted to be between 70 ± 5°F and 30 to 70%, respectively. Lighting was set to maintain an approximate 12-h light/dark cycle. Food (Certified Rodent Diet 5002; Nestle Purina PetCare Co., St. Louis, MO) 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* (Institute of Laboratory Animal Resources, 1996). 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 or 8 ml/kg. Blood was collected into lithium heparin blood tubes at various time points via retro-orbital bleeding under general anesthesia (80% CO₂/20% O₂). A terminal blood sample was taken from the posterior vena cava after euthanasia using CO₂. The blood was separated into plasma by centrifugation and then aliquoted and stored at -80°C until analyzed.

In one study, male humanized mice were dosed via intraperitoneal 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 5th day, the mice received SN-38 via intravenous administration, and blood was collected at various time points as described previously (*n* = 2 animals per time point for each study). After the terminal bleed (last time point), livers were removed, rinsed in saline, blotted

TABLE 1

Intravenous formulation and time points for pharmacokinetic studies of SN-38, ezetimibe, and naloxone in wild-type and *Tg(UGT1^{A1*28})Ugt1(-/-)* mice (*n* = 2 animals/time point)

Compounds	Mouse Type	Intravenous Dose mg/kg	Formulation	Time Point Range
SN-38	WT	1	10% Ethanol/70% PEG-400/20% PBS	5 min–8 h
	WT	2	10% Ethanol/70% PEG-400/20% PBS	5 min–3 h
	<i>Tg(UGT1^{A1*28})Ugt1(-/-)</i>	1	10% Ethanol/70% PEG-400/20% PBS	5–45 min
	<i>Tg(UGT1^{A1*28})Ugt1(-/-)</i>	2	10% Ethanol/70% PEG-400/20% PBS	2.5 min–3 h
	<i>Tg(UGT1^{A1*28})Ugt1(-/-)</i> with pb treatment	1	10% Ethanol/70% PEG-400/20% water	3–80 min
Ezetimibe	WT	1	10% Ethanol/50% PEG-400/40% PBS	5 min–8 h
	WT	5	10% Ethanol/50% PEG-400/40% PBS	5 min–3 h
	<i>Tg(UGT1^{A1*28})Ugt1(-/-)</i>	1	10% Ethanol/50% PEG-400/40% PBS	5–45 min
	<i>Tg(UGT1^{A1*28})Ugt1(-/-)</i>	5	10% ethanol/50% PEG-400/40% PBS	2.5 min–3 h
	Naloxone	5	10% NMP/50% PEG-400/40% PBS	5 min–8 h
Naloxone	WT	5	10% NMP/50% PEG-400/40% PBS	5 min–8 h
	<i>Tg(UGT1^{A1*28})Ugt1(-/-)</i>	5	10% NMP/50% PEG-400/40% PBS	5 min–8 h

WT, C57BL/6 mouse; PEG, polyethylene glycol; PBS, phosphate-buffered saline; NMP, *N*-methylpyrrolidone; pb, phenobarbital.

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 using an LC/MS/MS system composed of an SCL-10A VP Shimadzu (Kyoto, Japan) pump LC system controller, coupled with a triple quadrupole mass spectrometer using multiple reaction monitoring in the positive electrospray ionization mode (API4000; Applied Biosystems/MDS Sciex, Foster City, CA). The separation of the analytes was achieved by using an AQUASIL C18 $20 \times 2.1\text{-mm}$ ($3 \mu\text{m}$) (Thermo Fisher Scientific) 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 $393.0 \rightarrow 249.2$ at 1.08 min, $410.1 \rightarrow 132.9$ at 1.4 min, $328.25 \rightarrow 253.1$ at 0.95 min, and $237.0 \rightarrow 194.0$ at 1.28 min, respectively. Quantitation of the analytes was performed using Analyst 1.2 software (Applied Biosystems/MDS Sciex).

Pharmacokinetic calculations were performed using the noncompartmental approach with the aid of Watson LIMS (version 7.2.0.03; Thermo Fisher Scientific). The initial plasma concentration (C_0) was determined by linear regression extrapolation from the first two 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 HLMs. All the 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 to 12% BisTris gels under denaturing conditions (50 mM MOPS, 50 mM Tris base, pH 7.7, 0.1% SDS, 1 mM EDTA) before transferring the proteins to a polyvinylidene difluoride membrane using a semidry transfer system (Invitrogen). 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 Professor 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 enhanced chemiluminescence plus Western blotting detection system (GE Healthcare, Waukesha, WI), and the proteins were detected after exposure to X-ray film.

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction Analysis. Pooled liver tissues were pulverized in liquid nitrogen, and approximately 100 mg of tissue was homogenized into 1 ml of TRIzol (Invitrogen). Two hundred microliters of chloroform was added with vigorous 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 isopro-

panol, collected by centrifugation, and followed by 75% ethyl alcohol wash. The RNA was dissolved into diethyl pyrocarbonate water, and the concentration was determined. Using Iscript Reverse Transcriptase (Bio-Rad Laboratories, 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 amplifications of human UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9, as described previously (Chen et al., 2005). In brief, the polymerase chain reaction with 1 μl of cDNA and 0.25 μM of each of the UGT1A-specific oligonucleotide primer pairs 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 min followed by 30 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. After the reaction, 15 μl of the polymerase chain reaction products was subjected to electrophoresis in 1.5% agarose gels and documented by ChemiDoc XRS gel imaging system (Bio-Rad Laboratories).

Results

Mouse Pharmacokinetic Studies. Pharmacokinetic parameters were determined in mice (intravenous route) for three established UGT substrates: SN-38, the active metabolite of CPT-11 that is cleared predominantly by UGT1A1; ezetimibe (Zetia), which is believed to form the phenolic glucuronide by UGT1A1, UGT1A3, and UGT2B15 (ezetimibe also forms a very minor benzylic glucuronide by UGT2B7, which is not monitored in this report); and naloxone, which is a UGT2B7 substrate (Fig. 1). In the case of SN-38, at the doses tested, no adverse effect was seen with either wild-type C57BL/6 or *Tg(UGT1^{A1*28})Ugt1(-/-)* mice. The pharmacokinetic results are summarized in Table 2 and displayed in Fig. 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 i.v. dose, SN-38 exhibited a clearance of 320 ml/min/kg and an AUC of 0.14 $\mu\text{M}\cdot\text{h}$, whereas at the 2 mg/kg i.v. dose, the clearance was lower and the exposure was higher (clearance of 230 ml/min/kg and an AUC of 0.37 $\mu\text{M}\cdot\text{h}$, respectively). For ezetimibe, 1 mg/kg i.v. yielded a clearance value of 136 ml/min/kg and an AUC of 0.30 $\mu\text{M}\cdot\text{h}$. At higher doses (5 mg/kg), clearance was constant, whereas the AUC increased approximately 5-fold. Naloxone, at 5 mg/kg i.v., had a high clearance of 194 ml/min/kg and an AUC of 1.3 $\mu\text{M}\cdot\text{h}$.

Humanized UGT1 Mouse Studies. The development of *Tg(UGT1^{A1*28})Ugt1(-/-)* mice leads to minimal expression of hepatic UGT1A1 (Fig. 3). In 8-week-old mice, the serum bilirubin levels are mildly elevated compared with same-aged 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

FIG. 1. Glucuronidation reactions investigated in this study.

TABLE 2

Pharmacokinetic parameters of SN-38, ezetimibe, and naloxone in wild-type C57BL/6 and *Tg(UGT1^{A1*28})Ugt1(-/-)* mice (dosed intravenously, *n* = 2 mice/time points; values in parentheses represent individual determinations)

Compounds	Mouse Type	Intravenous Dose	AUC	CL
		mg/kg	$\mu\text{M} \cdot \text{h}$	ml/min/kg
SN-38	WT	1	0.14 (0.15, 0.12)	320 (276, 363)
	WT	2	0.37 (0.34, 0.39)	230 (241, 219)
	Humanized	1	0.54 (0.57, 0.50)	80 (74, 86)
	Humanized	2	0.87 (0.79, 0.95)	96 (100, 92)
	Humanized with pb treatment	1	0.15 (0.18, 0.11)	285 (240, 330)
Ezetimibe	WT	1	0.30 (0.31, 0.29)	136 (131, 141)
	WT	5	1.7 (1.67, 1.74)	120 (122, 117)
	Humanized	1	0.31 (0.28, 0.35)	132 (145, 118)
	Humanized	5	3.3 (3.60, 3.09)	63 (59, 66)
Naloxone	WT	5	1.3 (1.2, 1.4)	194 (197, 191)
	Humanized	5	1.2 (1.3, 1.1)	210 (199, 221)

CL, clearance; WT, C57BL/6 mouse; pb, phenobarbital.

glucuronidation would be expected to show differences in clearance and AUC. For SN-38, the AUC increased from 0.54 to 0.87 $\mu\text{M} \cdot \text{h}$ 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 before administration of SN-38, hepatic UGT1A1 expression was greatly induced (Fig. 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 after phenobarbital treatment (Fig. 4). Consistent with the induction of UGT1A1, clearance of SN-38 in phenobarbital-treated mice increased (96–285 ml/min/kg), an observation that is concordant with a reduction in AUC (0.87 to 0.15 $\mu\text{M} \cdot \text{h}$).

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(UGT1^{A1*28})Ugt1(-/-)* mice with wild-type mice. At a higher dose of ezetimibe (5 mg/kg), small differences (approximately 2-fold) in pharmacokinetic parameters were seen between *Tg(UGT1^{A1*28})Ugt1(-/-)* and wild-type mice. Finally, naloxone pharmacokinetics were evaluated, and the AUC and

clearance values were nearly identical when comparing results between wild-type and *Tg(UGT1^{A1*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 HLMs genotyped for the normal (*UGT1A1*1*) and the Gilbert's (*UGT1A1*28*) allele, in addition to MLMs prepared from untreated wild-type mice, *Tg(UGT1^{A1*28})Ugt1(-/-)* mice, and phenobarbital-treated *Tg(UGT1^{A1*28})Ugt1(-/-)* 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 Fig. 5 (for HLM) and Fig. 6 (for MLM).

Enzyme Kinetics with HLM. Four individual lots of HLM were obtained commercially from BD Gentest. They were genotyped in-house, and Western blot analysis was also performed (Fig. 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

FIG. 2. Time courses of SN-38, ezetimibe, and naloxone in mice dosed intravenously (n of 2 mice/time point, and each data point shown represents the average of two determinations). Diamonds, wild-type C57BL/6 mice; squares, humanized *UGT1* mice; triangles, humanized *UGT1* mice with phenobarbital treatment.

FIG. 3. Protein expression of UGT1A1 in *Tg(UGT1A1*28)Ugt1(-/-)* male MLMs and HLMs. Mice were treated with phenobarbital at 100 mg/kg/day for 4 days. Left, control = *Tg(UGT1A1*28)Ugt1(-/-)* mice without phenobarbital treatment; pb, *Tg(UGT1A1*28)Ugt1(-/-)* mice with phenobarbital treatment. HLMs have been genotyped for expression of the *UGT1A1*28* or *UGT1A1*1* alleles.

FIG. 4. Human *UGT1A* gene expression in *Tg(UGT1A1*28)Ugt1(-/-)* mice treated with phenobarbital. Six age-matched humanized *Tg(UGT1A1*28)Ugt1(-/-)* mice were treated intraperitoneally with either dimethyl sulfoxide (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 reverse transcription-polymerase chain reaction to determine the expression levels of human *UGT1A1*, *UGT1A3*, *UGT1A4*, *UGT1A6*, and *UGT1A9*.

protein in each of the microsomal preparations of *UGT1A1*28* origin. For the kinetic analysis with SN-38, the K_m ranged from 0.635 to 1.46 μM , whereas $V_{\max}$ decreased 4- to 8-fold between HLM genotyped for the *UGT1A1*1* and *UGT1A1*28* alleles (Table 3).

For ezetimibe, K_m ranged from 5.18 to 16.9 μM and $V_{\max}$ 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_m (ranging 34.5–63.1 μM) or $V_{\max}$ (ranging 1593–2059 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(-/-)* mice in addition to *Tg(UGT1A1*28)Ugt1(-/-)* mice treated with phenobarbital are provided in Table 3. For SN-38, the K_m values ranged from 1.44 μM in MLM prepared from phenobarbital-treated *Tg(UGT1A1*28)Ugt1(-/-)* mice to 3.59 μM in wild-type MLM. The $V_{\max}$ increased from 43.6 pmol/min·mg in untreated *Tg(UGT1A1*28)Ugt1(-/-)* mice to 497 pmol/min·mg in MLM prepared from phenobarbital-treated *Tg(UGT1A1*28)Ugt1(-/-)* mice (~11-fold), a finding that supports the robust induction of *UGT1A1* in microsomes from phenobarbital-treated *Tg(UGT1A1*28)Ugt1(-/-)* mice. When ezetimibe and naloxone were evaluated, there were no differences in K_m and $V_{\max}$ values using microsomes from untreated and phenobarbital-treated *Tg(UGT1A1*28)Ugt1(-/-)* mice.

Discussion

Given the importance of P450 and UGTs 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). In addition, 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). On the other hand, 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. It is clear that 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 because the humanization process does not alter expression of the constitutive mouse-specific *UGT2* enzymes, as we have shown 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 CPT-11 that is cleared predominantly by *UGT1A1*; ezetimibe (Zetia), which is reported to be eliminated partially by *UGT1A1*; and naloxone, which is not a *UGT1A1* substrate (Fig. 1). CPT-11 and SN-38 have been well characterized because of their adverse effect (diarrhea) in some patients (Iyer et al., 1998, 1999; Onoue and Inui, 2008). After intravenous administration of a 1-mg/kg

TABLE 3

Kinetic parameters of glucuronidation from incubations with individual HLMs and pooled MLMs (minimal n = 3 animals and triplicate determinations per data point)

Liver Microsomes	SN-38		Ezetimibe		Naloxone	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
	μM	$pmol/min \cdot mg$	μM	$pmol/min \cdot mg$	μM	$pmol/min \cdot mg$
Human liver microsomes						
HH83 (WT)	0.964 ± 0.274	162 ± 10.9	5.18 ± 2.28	781 ± 85.9	58.5 ± 6.85	1593 ± 50.1
HH112 (WT)	0.680 ± 0.217	88.2 ± 6.73	14.7 ± 4.43	1515 ± 146	54.6 ± 6.49	2059 ± 67.6
HH9 (*28/*28)	1.46 ± 0.637	21.0 ± 2.37	16.9 ± 3.62	1619 ± 116	34.5 ± 6.66	1882 ± 91.3
HH81 (*28/*28)	0.635 ± 0.253	17.6 ± 1.51	11.5 ± 3.47	1330 ± 120	63.1 ± 15.7	1986 ± 149
Mouse liver microsomes						
WT C57BL/6 mouse	3.59 ± 1.21	201 ± 21.5	15.0 ± 3.11	859 ± 64.5	278 ± 23.6	1635 ± 55.7
$Tg(UGT1A1^{*28})Ugt1(-/-)$	2.42 ± 1.23	43.6 ± 6.36	35.6 ± 2.33	1093 ± 31.7	363 ± 39.3	1787 ± 84.1
$Tg(UGT1A1^{*28})Ugt1(-/-)$ with pb treatment	1.44 ± 0.359	497 ± 28.8	20.0 ± 3.31	1025 ± 65.8	432 ± 31.6	1723 ± 58.1

WT, C57BL/6 mouse; pb, phenobarbital.

FIG. 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/data points). Open circles, HH83; open triangles, HH112; open squares, HH9; open diamonds, HH81.

dose in mice, SN-38 showed approximately 4-fold differences in parent drug exposure (AUC) and clearance in the humanized mice compared with wild-type mice. The extremely high clearance values

for SN-38 were likely caused by 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. Because *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. In addition, 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; Gagné 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 CPT-11 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 showed similar trends as those determined in HLM. In particular, 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 compared with 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. (2004) previously showed ezetimibe to be a substrate for multiple human UGT forms (*UGT1A1*, *UGT1A3*, *UGT2B7*, and *UGT2B15*). Our investigation began with mouse pharmacokinetic determinations and with ezetimibe administration to wild-type and *Tg(UGT1A1*28)Ugt1(-/-)*

FIG. 6. Substrate concentration-dependent glucuronidation of SN-38 (A), ezetimibe (B), and naloxone (C) by MLMs prepared from wild-type C57BL/6 mice (open circles), *Tg(UGT1^{A1}*28)Ugt1(-/-)* mice without phenobarbital treatment (open squares), and with phenobarbital treatment (open triangles) (*n* of 3 replicates/data points using pooled MLMs prepared from a minimum of three animals).

mice at 1 mg/kg i.v.; 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 the result of enzyme (UGT1A1) saturation in *Tg(UGT1^{A1}*28)Ugt1(-/-)* mice (given the low protein levels; Fig. 3). Overall, this effect 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(UGT1^{A1}*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(UGT1^{A1}*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 *UGT1A1**1 or *UGT1A1**28. More importantly, the rate of ezetimibe glucuronide formation was not reduced in HLM samples genotyped as *UGT1A1**28 compared with normal

*UGT1A1**1 HLMs, indicating that glucuronidation by UGT1A1 may not play a major role in overall ezetimibe metabolism. Because 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-UGT1* mice (Fig. 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 depend on tissue-specific factors in addition to variations in heterodimer formation (Operañña and Tukey, 2007), may result in glucuronidation efficiencies that are much different from those observed in vitro using expressed forms of single UGTs.

In summary, the *Tg(UGT1^{A1}*28)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 *UGT1A1**1 allele can result in up to 10-fold greater protein concentration in liver compared with those that are homozygous for *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 *UGT1* mice to examine pharmacokinetic parameters in vivo, especially when substrates are selectively metabolized by UGT1A1. Recent experiments using transgenic *UGT1* mice have been used successfully to examine the pharmacokinetic parameters of lamotrigine, a popular antiepileptic 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 underpredict 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) toward providing accurate information in predicting human drug metabolism.

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