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Common Human *UGT1A* Polymorphisms and the Altered Metabolism of Irinotecan Active Metabolite 7-Ethyl-10-hydroxycamptothecin (SN-38)

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ABSTRACT

7-Ethyl-10-hydroxycamptothecin (SN-38) is the pharmacologically active metabolite of irinotecan, in addition to being responsible for severe toxicity. Glucuronidation is the main metabolic pathway of SN-38 and has been shown to protect against irinotecan-induced gastrointestinal toxicity. The purpose of this study was to determine whether common polymorphic UDP-glucuronosyltransferase (UGT) affects SN-38 glucuronidation. First, kinetic characterization of SN-38-glucuronide (SN-38-G) formation was assessed for all known human UGT1A and UGT2B overexpressed in human embryonic kidney 293 cells. To assess the relative activity of UGT isoenzymes for SN-38, rates of formation of SN-38-G were monitored by liquid chromatography/mass spectrometry analysis and normalized by level of UGT cellular expression. Determination of intrinsic clearances predicts that hepatic UGT1A1 and UGT1A9 and the extrahepatic UGT1A7 are major components in SN-38-G formation, whereas a minor role is suggested for UGT1A6, UGT1A8, and UGT1A10. In support of the involvement of UGT1A9, a strong coefficient of correlation was observed in the glucuronidation of SN-38 and a substrate, mainly glucuronidate, by UGT1A9 (flavopiridol) by human liver microsomes (coefficient of correlation, 0.905; $p = 0.002$). In vitro functional experiments revealed a negative impact of the UGT1A1 allelic variants. Residual activities of 49, 7, 8, and 11% were observed for UGT1A1*6 ($G^{71}R$), UGT1A1*27 ($P^{229}Q$), UGT1A1*35 ($L^{233}R$), and UGT1A1*7 ($Y^{486}D$), respectively. Common variants of UGT1A7, UGT1A7*3 ($N^{129}K;R^{131}K;W^{208}R$), and UGT1A7*4 ($W^{208}R$), displayed residual activities of 41 and 28% compared with the UGT1A7*1 allele. Taken together, these data provide the evidence that molecular determinants of irinotecan response may include the *UGT1A* polymorphisms studied herein and common genetic variants of the hepatic UGT1A9 isoenzyme yet to be described.

Clinical trials have established irinotecan in combination with 5-fluorouracil/leucovorin as the new standard of care in the first-line treatment of metastasised colorectal cancer (Saltz et al., 2000, 2001; Cunningham et al., 2001; Rothenberg, 2001). However, severe dose-limiting toxicities are associated with irinotecan treatment; the most common are diarrhea, leukopenia, and myelosuppression. Recent evidence supports the idea that the metabolizing capacity of cancer patients is a major determinant factor of irinotecan-associated toxicity (Mani, 2001; Ratain, 2002).

Irinotecan [7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin (CPT-11)] is a potent inhibitor of topoisomerase I, which is biotransformed to an active metabolite by carboxylesterases to 7-ethyl-10-hydroxycamptothecin (SN-38) (Kawato et al., 1991). SN-38 undergoes significant glucuronidation to form the corresponding inactive glucuronide [10-*O*-glucuronyl-SN-38 (SN-38-G)] (Gupta et al., 1994). The major dose-limiting toxicity of irinotecan therapy diarrhea is suggested to be secondary to the biliary excretion of SN-38, the extent of which is determined by SN-38 glucuronidation (Rothenberg, 1998). Accordingly, recent findings indicate that the metabolism of irinotecan via glucuronidation protects against irinotecan-induced gastrointestinal toxicity. An inverse relationship was observed between SN-38 glucuronidation rates and severity of diarrheal incidences in patients treated with irinotecan (Gupta et al., 1994; Iyer et al., 1999).

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ABBREVIATIONS: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy camptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38-G, 7-ethyl-10-hydroxycamptothecin glucuronide; UGT, UDP-glucuronosyltransferase enzymes; HEK, human embryonic kidney; ECL, enhanced chemiluminescence.

Inherited differences in irinotecan glucuronidating capacity may have an important influence on the pharmacokinetics, pharmacologic effects, and toxicity of this drug because glucuronidation is the major route of detoxification and elimination of the active metabolite SN-38. Important interindividual variability was observed in irinotecan pharmacokinetics among cancer patients (Rivory and Robert, 1994; Iyer, 1999). Several mechanisms could explain this variability and include the presence of genetic polymorphisms in the metabolizing capacity of cancer patients. Hepatic metabolism and biliary excretion are major elimination pathways of irinotecan. Key enzymatic processes are involved in the biotransformation of CPT-11 and its metabolites, including carboxylesterase enzymes, which convert CPT-11 to SN-38; CYP3As, which convert CPT-11 into 7-ethyl-10[4-*N*-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin and 7-ethyl-10[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin; UGT enzymes, which convert SN-38 to SN-38-G, and bacterial β-glucuronidases, which are responsible for the deconjugation of SN-38-G in the intestinal tract (Mathijssen et al., 2001). The present study has focused on the biotransformation of SN-38 active metabolite into the β-D-glucuronide derivative SN-38-G by human uridine diphospho-glucuronosyltransferase enzymes (UGT).

UGT1A1 isoenzyme was suggested to be the predominant human UGT involved in the formation of SN-38-G. A strong correlation between UGT1A1 low (*UGT1A1*28*) and high (*UGT1A1*1*) promoter glucuronidating alleles and rates of glucuronidation of SN-38 was observed (Iyer et al., 1999). In addition, a clinical study revealed the importance of this genetic lesion in the *UGT1A1* gene and its association with the occurrence of severe irinotecan-induced toxicity (Ando et al., 2000). Therefore, the main purpose of the present study was to determine the effect of additional common inherited variations in specific *UGT* genes and which enzyme products are involved in SN-38 glucuronidation.

To date, the multigene superfamily of human UGT includes more than 24 genes and cDNAs, 16 of which are functional. Based on their sequence similarities, UGTs have been grouped into two gene families: *UGT1* and *UGT2* and four subfamilies, *UGT1A*, *UGT2A*, *UGT2B*, and *UGT2C*. The structure of seven *UGT2B* genes and cDNA have been published (Mackenzie et al., 1997; Riedy et al., 2000; Levesque et al., 2001), in addition to five homologous pseudogenes (Turgeon et al., 2000). In contrast to the *UGT2B* family, which comprises several genes, the entire UGT1 family is derived from a single gene locus (*UGT1A*) located on chromosome 2 (2q37), coding for nine functional proteins (UGT1A1, UGT1A3–1A10) and four pseudogenes (p) (UGT1A2p, UGT1A11p, UGT1A12p and UGT1A13p) (Gong et al., 2001). Currently, several common genetic polymorphisms in UGT enzymes have been described and their effects on SN-38 glucuronidation never explored.

In the present study, we first aimed to determine the identity and the relative contribution of human UGT isoenzymes in the formation of SN-38-G. For the first time, all 16 UGT1A and UGT2B recombinant proteins isolated to date were included and their capacity to form SN-38-G was determined in the same experimental conditions. Then, the potential effect of common genetic variations in UGT relevant to SN-38 glucuronidation was determined, after the production of HEK293 stable cell lines overexpressing variant UGT proteins. Functional polymorphisms have been described previously for two UGT1A enzymes, UGT1A1 and UGT1A7, which have demonstrated reactivity toward SN-38. Our results demonstrate that a second hepatic UGT, UGT1A9, is involved in SN-38-G formation, in addition to UGT1A1. Furthermore, the significant decrease in SN-38-G formation associated with common polymorphic variants of UGT1A1 and UGT1A7 observed herein, suggests that patients with these UGT genetic abnormalities could be at increased risk of developing severe irinotecan-associated toxicity.

Experimental Procedures

Materials. SN-38 was kindly provided by Dr. James Patrick McGovern (Pharmacia & Upjohn, Inc., Kalamazoo, MI). UDP-glucuronic acid was obtained from Sigma Chemical Co. (Oakville, Ontario CA). All other chemical and reagents were of the highest grade available.

Stable Expression of Human *UGT1A* and *UGT2B* Alleles. The isolation of human UGT1A and UGT2B cDNAs and their stable expression in HEK293 have been described previously (Albert et al., 1999; Guillemette et al., 2000; Levesque et al., 2001; Turgeon et al., 2001). To prepare HEK cell populations stably transfected with each of the cDNA encoding alleles of UGT1A1 and UGT1A7, expression vectors encoding variants were prepared using a Quickchange polymerase chain reaction site-directed mutagenesis kit (Stratagene, La Jolla). *UGT1A1*1* and *UGT1A7*1* were used as the starting pcDNA3 plasmids. All mutations investigated in the present study were published previously, whereas the novel UGT1A1 allele will be described elsewhere and was assigned *UGT1A1*35* (GenBank/EMBL accession no. AF110194), based on the nomenclature system proposed by Mackenzie et al. (1997). Polymerase chain reactions were performed as described previously (Guillemette et al., 2000) using forward and reverse primers listed in Table 1. An expression vector carrying the desired mutation(s) was obtained and the entire sequence of the cDNA was verified by sequencing. HEK cells were transfected as described previously (Guillemette et al., 2000) using LipofectAMINE (Invitrogen, Carlsbad, CA). Forty-eight hours after transfections, G418 (1 mg/ml) was added to select UGT-expressing cell populations.

Characterization of HEK293 Cell Systems Expressing UGT1A and UGT2B and Their Genetic Variants. Microsomal proteins used for UGT expression and enzymatic activities were extracted as described previously (Guillemette et al., 2000). To ascertain the level of UGT protein expression in the stable UGT1A- and UGT2B- HEK293 clones, a semiquantitative immunoblot analysis method was used. For quantification of the UGT1A proteins, we used the anti-human UGT1A common carboxyl terminus region (amino acids 312 to 531) antiserum RC-71, as reported previously. The specificity of this antiserum was assessed by Western blot analysis and immunoblots demonstrating its specificity for UGT1A proteins (Albert et al., 1999). UGT2B protein levels were quantified using the anti-human UGT2B antibody (EL-93), as described previously (Guillemette et al., 1997). To normalize sample loading, blots were stripped and re-probed with anti-calnexin antibody (Stressgen Biotechnologies, Victoria, BC, Canada), to detect a second endoplasmic reticulum-resident protein, calnexin. Bands were visualized using enhanced chemiluninescence (ECL; Amersham Biosciences, Piscataway, NJ) and quantified by Bioimage Visage 110s from Genomic Solutions Inc (Ann Harbor, MI).

SN-38 Glucuronidation Analysis by Electrospray/Ion-Trap MS. A liquid chromatographic/tandem mass spectrometric method was developed to quantify SN-38 glucuronidation of UGT cell line-derived microsomes and human liver microsomes (Fig 1). Samples were analyzed using high performance liquid chromatography (Alliance 2690; Waters, Milford, MA). Chromatographic separation was achieved with a Colombus C18 column 5-μm packing material, 50 × 3.2 mm (Phenomenex, Torrance, CA) using a two-solvent gradient

system: A (water + 1 mM ammonium formate); B (MeOH + 1 mM ammonium formate). At a constant flow rate (0.7 ml/min), a linear gradient from 20 to 65% B was run over 3 min, held 0.8 min and a second gradient until 95% of eluent B was run over 2 min and then re-equilibrated to 20% B over 2 min. The effluent from the high-performance liquid chromatography system (Alliance 2690) was connected directly to a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray ionization source and operated in positive ion mode. The data was acquired in two events. First, the mass spectrometer was operated in full scan MS and the SN-38 ion (393 *m/z*) was monitored. Second, full scan tandem MS was used to obtain the ion corresponding to the SN-38 glucuronide (569 *m/z*). Retention times for SN-38-G and SN-38 were 2.50 and 3.72 min, respectively.

Human Liver Microsomes. Eight individual human liver samples and one pool were included in the study to assessed the inter-

TABLE 1
Primers used for directed mutagenesis
Bold letters represent mutations produced by PCR-based mutagenesis

Primers		Sequences
UGT 1A1		
**27*	A	5′-cgtggtttattccc**a**gtatgcaacccttg-3′
	B	5′-caagggttgcatac**t**gggaataaaccacg-3′
**7*	C	5′-ctcacctggtacca**g**gaccattccttggacg-3′
	D	5′-cgtccaaggaatggtc**c**tggtaccaggtgag-3′
**6*	E	5′-cgcctcgttgtacatcagagac**a**gagcattttacaccttg-3′
	F	5′-caaggtgtaaaatgctc**t**gtctctgatgtacaacgaggcg-3′
**35*	G	5′-ttattccccgtatgcaaccc**t**tgcctcagaattccttcaga-3′
	H	5′-tctgaaggaattctgaggca**a**gggttgcatacggggaataa-3′
UGT 1A7		
**2*	I	5′-caaggagagagta**t**ggaaccacatcatgcac-3′
	J	5′-gtgcatgatgtggttcc**a**tactctctccttg-3′
**4*	K	5′-ggagtttgtttaa**t**gac**cg**aaaattagtag-3′
	L	5′-ctactaattt**tcg**gtc**a**ttaaacaaactcc-3′

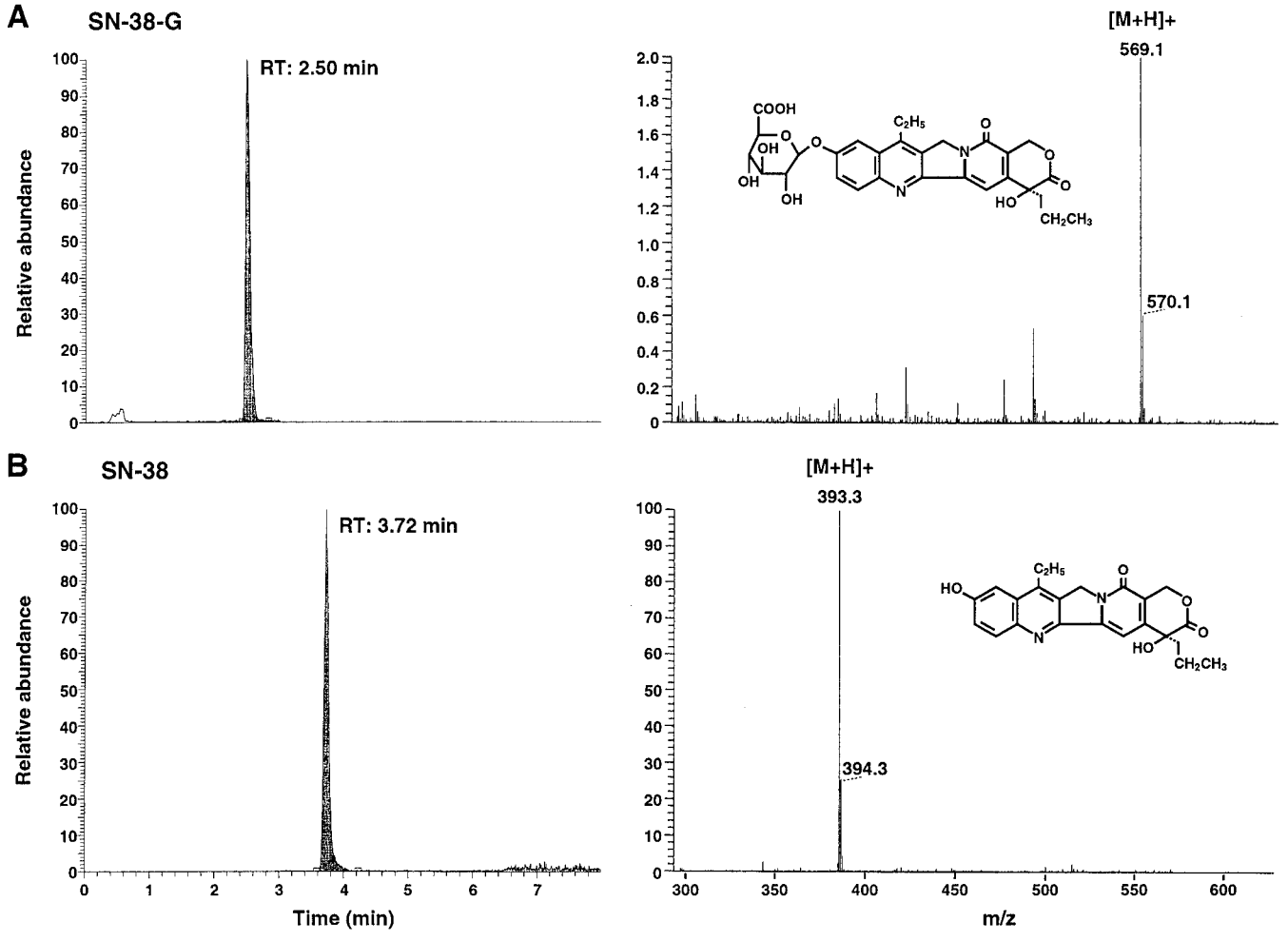

Fig. 1. Detection of SN-38 glucuronide formation by LCMS. LCMS spectra of SN-38 (B) and SN-38-G (A) are shown. Molecular weights were determined by mass spectrometry for SN-38 and SN-38-G. Analysis of incubation mixtures with human liver microsomes revealed an additional peak with retention time of 2.50 min corresponding to SN-38-G. SN-38-G metabolite was observed at *m/z* 569.1 $[SN\text{-}38\text{-}G+H]^+$ (A). Analysis of a SN-38 standard showed a positive ion mass spectra similar to B, confirming that the peak eluting at 3.73 min with *m/z* 393.3 corresponds to $[SN\text{-}38+H]^+$.

individual variability of SN-38-G formation and determine UGT expression in human liver samples. The mean age of the liver donors was 50 years (34–65 years) and all were female white persons. Several cytochrome P450-associated activities were measured in these microsome samples, supporting the integrity of the samples which were obtained from Human Cell Culture Center Inc. (Laurel, MD). The protein content of the microsomal preparation was determined by the bicinchoninic acid method.

Glucuronidation Assay with SN-38. The microsomal fractions from UGT-HEK293 cells were used in enzymatic assays, whereas, in the case of UGT1A10, commercial microsomal fraction of Sf-9 insect cells infected with a baculovirus strain containing human 1A10 cDNA were used (Panvera, Madison, WI). Reactions (100-μl volume) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 μg/ml phosphatidylcholine, 8.5 mM saccharolactone, 2 mM UDP-glucuronic acid, 40 to 60 μg of membrane protein, and SN-38 as substrate (up to 400 μM). Time-course experiments were designed to determine the linearity of the glucuronidation reaction. Kinetic properties of individual UGT1A proteins were assessed and compared with human liver microsomes. For determination of V_{max} and K_m, HEK293 cells stably expressing UGT enzymes were incubated in the presence of varying SN-38 concentrations from 1 to 100 μM for the corresponding period of 1 h for UGT1A3, 3 h for UGT1A8, and 5 h for UGT1A1, UGT1A6, UGT1A7, UGT1A9, and UGT1A10. All reaction rates were shown to be linear for these times. Human liver microsomes were incubated under the same conditions for 1 h. Because of the lack of apparent enzyme latency, inclusion of detergent was found to be unnecessary for assessment of the full glucuronidating potential of *UGT1*-expressing HEK cell membranes, whereas alamethicin (0.2 mg/ml) (Sigma-Aldrich Chemical Co., Oakville, ON, Canada) was added for assays with liver microsomes (Fisher et al., 2000). Relative glucuronidation activities of UGT1A variants for SN-38 were determined using two maximal concentrations of SN-38 (200 and 400 μM). Glucuronidation assays in liver samples using flavopiridol as substrate were performed as described previously (Ramirez et al., 2002). Relative glucuronidation activities for flavopiridol (5 and 7 glucuronides) were determined for 1 h using 100 μM of substrate and in the same experimental conditions as used for SN-38.

Statistical Analysis. Results were expressed as mean ± S.D. The Spearman rank correlation coefficient was used to test the magnitude of the correlation between the glucuronidation of SN-38 and flavopiridol (SAS institute, JMP version 4.0.2). Differences in kinetic parameters between *UGT* allelic variants were evaluated for statistical significance by paired Student's *t* test. All tests were two-sided.

Results

Formation of SN-38-G Catalyzed by Recombinant UGT Enzymes Expressed in HEK293 Cells. Previous report by the group of Iyer et al. (1998) demonstrated that UGT1A1 is the isoform responsible for SN-38 glucuronidation, although most of the human UGT isoenzymes were not tested individually. However, all UGT1A and UGT2B isoenzymes were not systematically tested in these studies. To determine the identify of the human UGT enzymes capable of forming the SN-38-G and to elucidate the relative contribution of individual UGT isoenzymes to SN-38 glucuronidation, reactivity of all sixteen known human UGT1A and UGT2B proteins characterized to date was assessed for SN-38.

Stable cell lines expressing human UGT1A and UGT2B proteins were produced using a unique human cell line, HEK293, which presents undetectable level of SN-38 UGT activity. Expression of UGT protein in selected clones was confirmed using specific UGT1A and UGT2B antibodies before enzymatic assays (Fig. 2). We assessed the ability of UGT isoenzymes to conjugate SN-38, using UGT1A1 and UGT1A3 through UGT1A9 and UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28-HEK293 cell lines. UGT1A10 baculosomes were used to determine reactivity toward SN-38, because of the low expression of UGT protein in our UGT1A10-HEK293 cell line compared with all other UGT-HEK293 cell lines (data not shown). UGT activities toward SN-38 were determined using a liquid chromatographic tandem mass spectrometric method (LC/MS/MS) (Fig. 1). For each cell lines, linearity of the glucuronidation reaction was first determined as described under *Experimental Procedures*. Conditions of the assays were selected to have formation of aglycone-glucuronide from substrate proportional to both time and protein concentration. SN-38 glucuronidation was observed after incubation with *UGT1*-encoded isoenzymes, namely UGT1A1, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10. SN-38-G could not be detected by LC/MS/MS after incubation with UGT1A3, UGT1A4, UGT1A5, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28 for up to 16 h at 37°C (data not shown). Results show that the UGT1A proteins are major in SN-38-G formation whereas UGT2B proteins would have little or no impact on the formation of glucuronide of SN-38.

Identification of the Major UGT1 Involved in the Formation of SN-38 Glucuronide. For a more comprehensive characterization of the glucuronidating activities of UGT1 isoenzymes, kinetic parameters were determined under the same experimental conditions. Apparent Michaelis kinetic constants (K_m) and maximal velocities (V_{max}) are presented in Table 2. Under linear kinetic conditions, results demonstrated a higher affinity for UGT1A1, UGT1A7, and UGT1A9 (apparent K_m = 7.5 ± 3.9; 1.2 ± 0.1; and 0.7 ± 0.2 μM), whereas UGT1A6, UGT1A8, and UGT1A10 demonstrated lower affinity (apparent K_m = 11.6 ± 5.2; 20.3 ± 4.5, and 31.5 ± 9.2 μM) compared with human liver microsomes (apparent K_m = 6.8 ± 3.0 μM). Absolute V_{max} values would indicate that UGT1A1 is the most efficient in the formation of SN-38-G compared with all other UGT1A active on SN-38. However, to evaluate accurately the glucuronidation efficiency of each UGT isoenzyme, the amount of expressed UGT protein was considered to determine the catalytic activity of SN-38 glucuronidation. Thus, to better assess the relative contribution of individual UGT enzyme in SN-38-G formation, glucuronidating activity toward SN-38 was normalized by the level of expressed UGT protein in recombinant UGT-HEK293 cells as determined by Western blot analyses (Fig. 2). The normalized V_{max} (relative V_{max}) was used to determined the efficiency of glucuronidation (intrinsic clearance or ratio V_{max}/K_m). As illustrated in Table 2, intrinsic clearances for SN-38 were high for two hepatic UGT, UGT1A1 (343 μl/h/mg), and UGT1A9 (315 μl/h/mg), and for the extrahepatic UGT1A7 isoenzyme (399 μl/h/mg). Lower catalytic efficiencies ranging from 3 to 11 μl/h/mg were observed for UGT1A6, UGT1A8, and UGT1A10.

Variability in the Formation of SN-38-G by Human Liver Microsomes and Correlation Studies Using a Main Substrate of UGT1A9. A previous study has demonstrated that the glucuronidation capacities of human liver samples are subjected to a wide interindividual variation in the hepatic formation of SN-38-G (Iyer et al., 1998). In the present study, we determined the level of variability in SN-38 glucuronide formation in a liver bank and assessed the

possible correlation with the level of UGT1A expressed protein because only members of this family showed reactivity for SN-38. Eight individual liver microsomal preparations were studied. Interindividual formation of SN-38-G was confirmed in our set of liver samples (mean value ± S.D. = 1901.1 ± 957.4; range 894.2–3725.7 pmol/h/mg; coefficient of variation of 50%) (Fig. 3A). UGT1A expression levels assessed by Western Blot were used to determine the possible correlation of SN-38 glucuronidation with the expression in UGT1A protein predicted to be major in the glucuronidation of SN-38 based on in vitro results described above (Fig. 3B). Results indicated that after normalization by the level of expressed protein, SN-38-G formation is still highly variable among individual subjects (5759.1 ± 2783.7 pmol/h/mg of UGT protein) (Fig. 3C). A weak correlation was observed between the level of UGT expression and the activity of SN-38 glucuronidation of liver samples ($r^2 = 0.03$). However, when removing sample number 2 and 6, a significant correlation between expression and activity was observed ($r^2 = 0.66$, $p = 0.005$).

To further assessed the possible involvement of the UGT1A9 hepatic isoenzyme in the metabolism of SN-38, the correlation between the glucuronidation of SN-38 and flavopiridol was determined in a subset of human liver samples. Flavopiridol is an anticancer drug in development and has recently been revealed as a specific substrate of UGT1A9

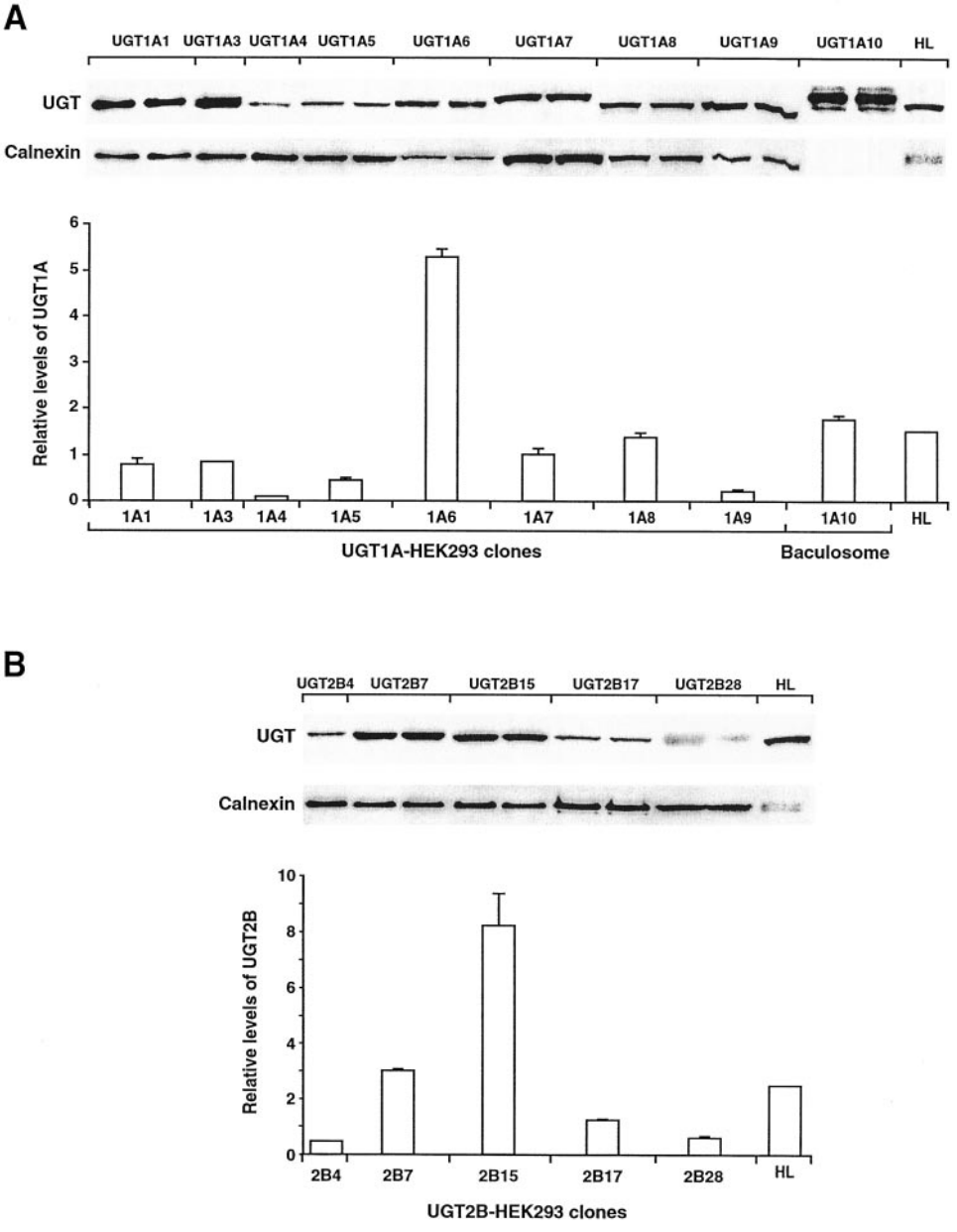

Fig. 2. Relative expression of UGT-HEK-293 selected clones. Microsomal proteins (20 μg protein loaded in each lane) HEK-293 cells expressing individual UGT1A (A) and UGT2B (B) isoenzymes were separated on SDS-PAGE, transferred onto nitrocellulose and probed with antisera raised against human UGT1A and UGT2B. To normalize sample loading, blots were stripped and reprobed to detect a second endoplasmic reticulum-resident protein, calnexin. Ratios between UGT and calnexin signals were calculated for each UGT except for UGT1A10 baculosome for which the UGT signal alone was considered for the expression level. The expression of UGT1A7 were arbitrarily designated as the basal UGT1A expression level. HL, human liver microsomes.

(Hagenauer et al., 2001; Ramirez et al., 2002). Flavopiridol glucuronidating activity determined by LCMS at 100 μM flavopiridol varied considerably between individuals ranging from 796.1 to 2672.6 pmol/h/mg of protein. A significant correlation was found between the in vitro formation of SN-38-G and flavopiridol-G by liver samples as determined by the Spearman rank correlation coefficient (0.905, $p = 0.002$), which was used to test the magnitude of the correlation between the glucuronidation of both compounds (Fig. 4), supporting that UGT1A9 would be involved in the hepatic glucuronidation of SN-38.

Catalytic Activities of Common Polymorphic UGT1A Isoenzymes for SN-38. We assessed the functional effect of known common allelic variations of UGT1A isoenzymes involved in the SN-38-G formation, namely UGT1A1 and UGT1A7. To evaluate the function of *UGT1A1*1* 'wild type' allele and $G^{71}R$ (*UGT1A1*6*), $Y^{486}D$ (*UGT1A1*7*), $P^{229}Q$ (*UGT1A1*27*), and $L^{233}R$ (*UGT1A1*35*) variant proteins of UGT1A1, we expressed all alleles in HEK293 cells. The *UGT1A1*35* allele was recently discovered in our laboratory and will be described elsewhere. The frequency of this allele was assessed in a population of white blood donors recruited in the Boston area (Guillemette et al., 2000). Only one patient was shown to be heterozygous for the *UGT1A1*35* allele and showed a leucine and an arginine at codon 233 ($L^{233}R$) in the exon 1 of UGT1A1 (data not shown), as confirmed by sequenced analysis. Among all normal blood donors that were genotyped, no additional patients were found to have the *UGT1A1*35* allele. Prevalence of other UGT1A1 polymorphisms associated with hyperbilirubinemia namely at codons 71, 229, and 486 of UGT1A1 were not determined in our population. The distribution of the previously described variants of UGT1A7, UGT1A7*2 ($N^{129}K$, $R^{131}K$), UGT1A7*3 ($N^{129}K$, $R^{131}K$, $W^{208}R$), and UGT1A7*4 ($W^{208}R$) were previously reported in the white population (Guillemette et al., 2000). Left of Fig. 5 show the Western blot analysis of microsomal preparations from selected clones of stably transfected HEK293 cells used for enzymatic assays and demonstrated significant UGT expression levels.

LC/MS/MS analyses of SN-38 metabolism showed that variant proteins of UGT1A1 produced significantly lower amounts of SN-38-G. Formation of SN-38-G by UGT1A1 variants at codons 71, 486, 229, and 233 was significantly reduced by 51, 93, 92, and 89% compared with UGT1A1*1 protein (Fig. 5A, right). Thus, it seems that the these UGT1A1 polymorphisms do result in an inactive UGT enzyme. Incubation of UGT1A7 microsomal proteins with SN-38 demonstrated that *UGT1A7*3* ($K^{129}K^{131}W^{208}$) and *UGT1A7*4* (W^{208}) present altered reactivity for SN-38, with a residual activity of 41 ($p<0.02$) and 28% ($p<0.002$), respectively, compared with the *UGT1A7*1* allele (Fig. 5B). No significant altered SN-38 glucuronidating activity was observed for *UGT1A7*2* compared with *UGT1A7*1*.

Discussion

The present study provides the first kinetic characterization of SN-38 glucuronidation by all known human UGT1A and UGT2B isoenzymes. Results revealed that two major hepatic UGT, UGT1A1 and UGT1A9, and the extrahepatic UGT1A7, are involved in SN-38 glucuronide formation. Effect of known UGT polymorphisms on SN-38-G formation was investigated in two UGT isoenzymes demonstrating reactivity for SN-38 and results predict a significant impact of common allelic variants of UGT1A1 and UGT1A7 on SN-38-G formation.

Recent findings indicate that the glucuronidation of SN-38 protects against irinotecan-induced gastrointestinal toxicity and led us to investigate the effects of common UGT polymorphisms on the in vitro glucuronidation of SN-38. As a first set of experiments, we determined the relative contribution of each of the sixteen UGT1A and UGT2B isoenzymes in the formation of inactive SN-38 glucuronide derivative using UGT-overexpressed HEK293 cell lines. Although previous studies have partially investigated the glucuronidation of SN-38 by UGT isoenzymes, none of them have systematically tested all of the existing UGT (Iyer et al., 1998; Ciotti et al., 1999). In addition, to better assess the relative contribution of individual UGT enzymes, the level of UGT protein expressed in the cell system was used to normalize SN-38 glucuronidating activities. Our data indicate that hepatic UGT1A9 presents an affinity 2- to 10-fold higher than UGT1A1 and UGT1A7. This is of particular interest because UGT1A9 like UGT1A1 is highly expressed in liver, the primary organ for detoxification of irinotecan. Comparison of the relative catalytic efficiencies of both hepatic UGT enzymes revealed very similar V_{max}/K_m ratios suggesting that the glucuronidation of SN-38 in human liver is catalyzed by both isoenzymes, with UGT1A9 having the highest affinity for the irinotecan active metabolite and UGT1A1 having the highest capacity. In contrast, a study by Ciotti and collaborators showed that UGT1A7 glucuronidates SN-38 at a higher level that of all others UGT1A isoenzymes (Ciotti et

TABLE 2

Apparent kinetic parameters for the glucuronidation of SN-38 by human liver microsomes (HLM) and human UGT isoenzymes

The values of apparent K_m and V_{max} for the formation of SN-38 glucuronide were determined using microsomal preparations from pool human livers and HEK293 cells. Formation of SN-38-G was measured by LC/MS/MS as described under *Experimental Procedures*. Values were expressed as the mean ± S.D. of two experiments performed in duplicate from Lineweaver-Burk plots.

UGT Source	Apparent K_m	Absolute V_{max}	Protein expression (relative to UGT1A7)	Relative V_{max}	Catalytic Efficiencies V_{max}/K_m
	μM	*pmol/h/mg protein*		*pmol/h/mg protein*	*μl/h/mg*
HLM	6.8 ± 3.0	2608.2 ± 442.0			
Hepatic and Gastrointestinal UGT					
UGT1A1	7.5 ± 3.9	2005.5 ± 124.7	0.78	2571.1 ± 159.9	343
UGT1A6	11.6 ± 5.2	163.5 ± 36.6	5.30	30.8 ± 6.9	3
UGT1A9	0.7 ± 0.2	145.7 ± 12.0	0.67	220.45 ± 17.9	315
Gastrointestinal UGT					
UGT1A7	1.2 ± 0.1	479.2 ± 11.1	1.00	479.2 ± 11.1	399
UGT1A8	20.3 ± 4.5	298.4 ± 45.0	1.38	216.2 ± 32.6	11
UGT1A10	31.5 ± 9.2	454.7 ± 24.3	1.77	256.9 ± 13.72	8

al., 1999). A detailed kinetic analysis was presented for UGT1A7 (Ciotti et al., 1999) and results of this study and the current work revealed similar apparent K_m. The discrepancy in results between the study of Ciotti et al. (1999) and the current study regarding the involvement of UGT1A1 and UGT1A9 in addition to UGT1A7, can be related to a number of factors including experimental conditions which varied considerably between studies in addition to the methods used to detect SN-38-G, thin layer chromatography versus mass spectrometry. In our conditions, we observed that results

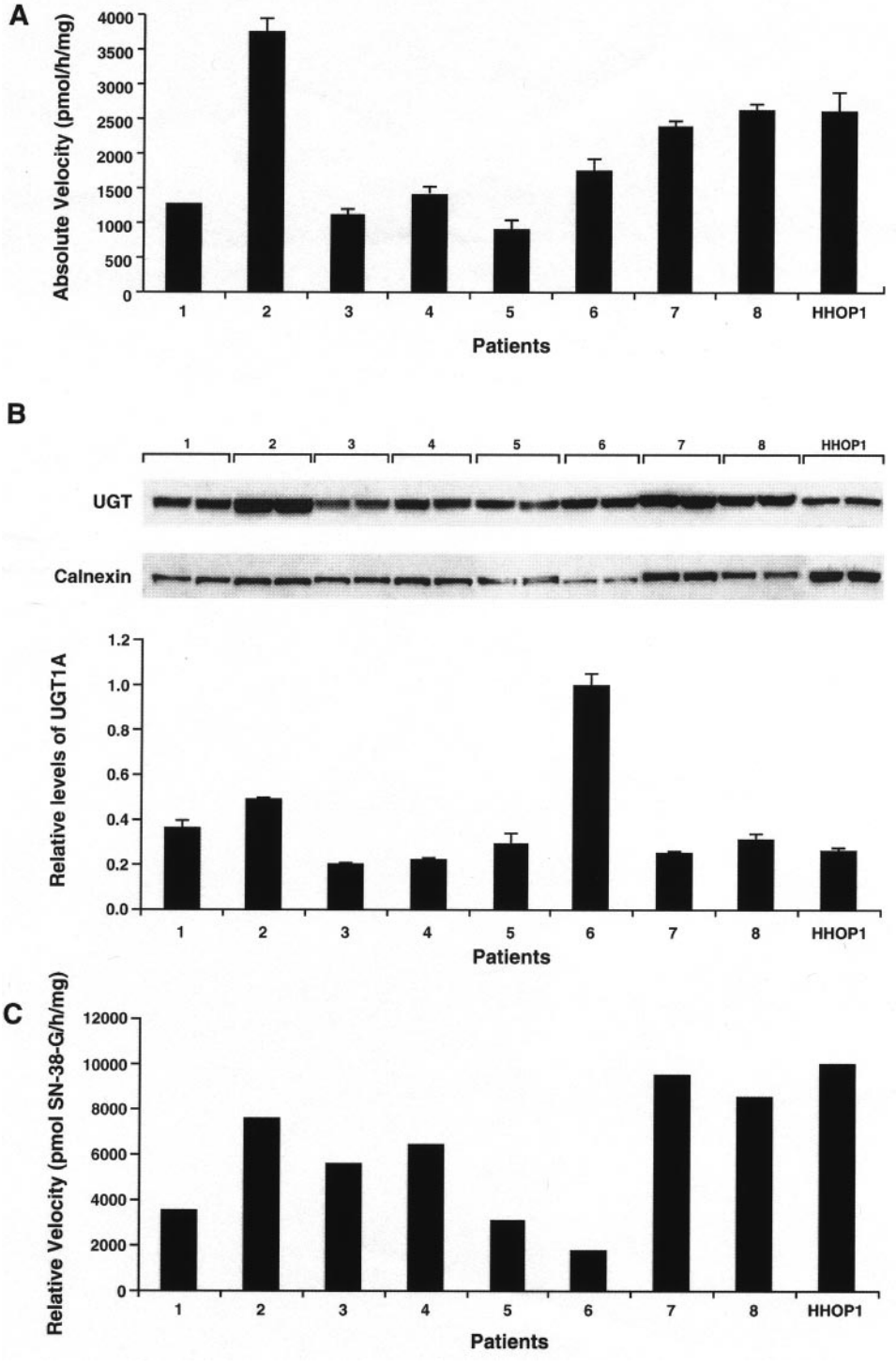

Fig. 3. Relative expression of the UGT1A proteins and SN-38 glucuronidating activities of human liver samples. Membrane fractions of nine liver samples, including a pool (20 μg of protein loaded in each lane) were subjected to SDS-PAGE followed by electrotransfer onto nitrocellulose and immunoblot analysis with antisera raised against human UGT1A. Ratios between the UGT and the calnexin signals were calculated for each liver samples. The expression of liver sample 6 was arbitrarily designated as the basal UGT1A expression level. A, relative levels of UGT1A determined by semiquantitative densitometric analysis of the ECL image. B, SN-38 glucuronidating activities by liver microsomes and determined by LCMS. C, relative activities after normalization by the corresponding levels of expressed UGT1A proteins. Values of formation of SN-38-G represent the mean ± S.D. of two experiments performed in duplicate. HHOP1; pool of human liver microsomes.

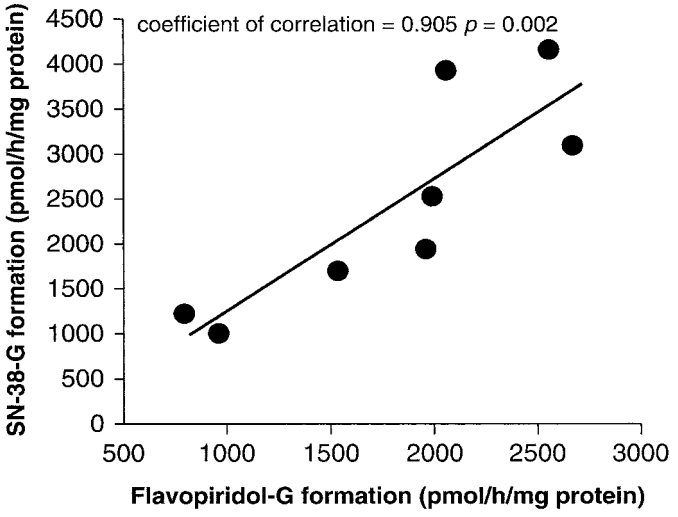

Fig. 4. Correlation analysis between the formation rates of SN-38-G and flavopiridol-G in eight human liver microsomes. Spearman coefficient of correlation of 0.905 ($p = 0.002$).

were more reproducible using LCMS rather than TLC. UGT1A1 was first identified as the main UGT involved in the glucuronidation of SN-38 (Iyer et al., 1998). Results of the involvement of UGT1A1 was based on in vitro glucuronidation studies with human UGT1A1 overexpressed in HEK293 cells and evidence of a strong correlation between the glucuronidation of SN-38 and bilirubin, a specific UGT1A1 substrate (Iyer et al., 1998). Herein, the contribution of UGT1A9 in the hepatic glucuronidation of SN-38 is suggested by a significant correlation of the SN-38-G and flavopiridol-G formation, a substrate mainly glucuronidated by UGT1A9 (Ramirez et al., 2002). The coefficient of correlation obtained was similar to the level of correlation previously observed between the formation of bilirubin-G and SN-38-G ($r^2 = 0.89$, $p = 0.001$) (Iyer et al., 1998). These results support a major role for UGT1A9 in the hepatic glucuronidation of SN-38, in addition to UGT1A1. However, the group of Hagenauer et al. (2001), also reported UGT1A1 as a main UGT involved in the glucuronidation of flavopiridol although in this study, levels of UGT protein expressed in the in vitro system were not used to normalize glucuronidation activities. Thus, the rela-

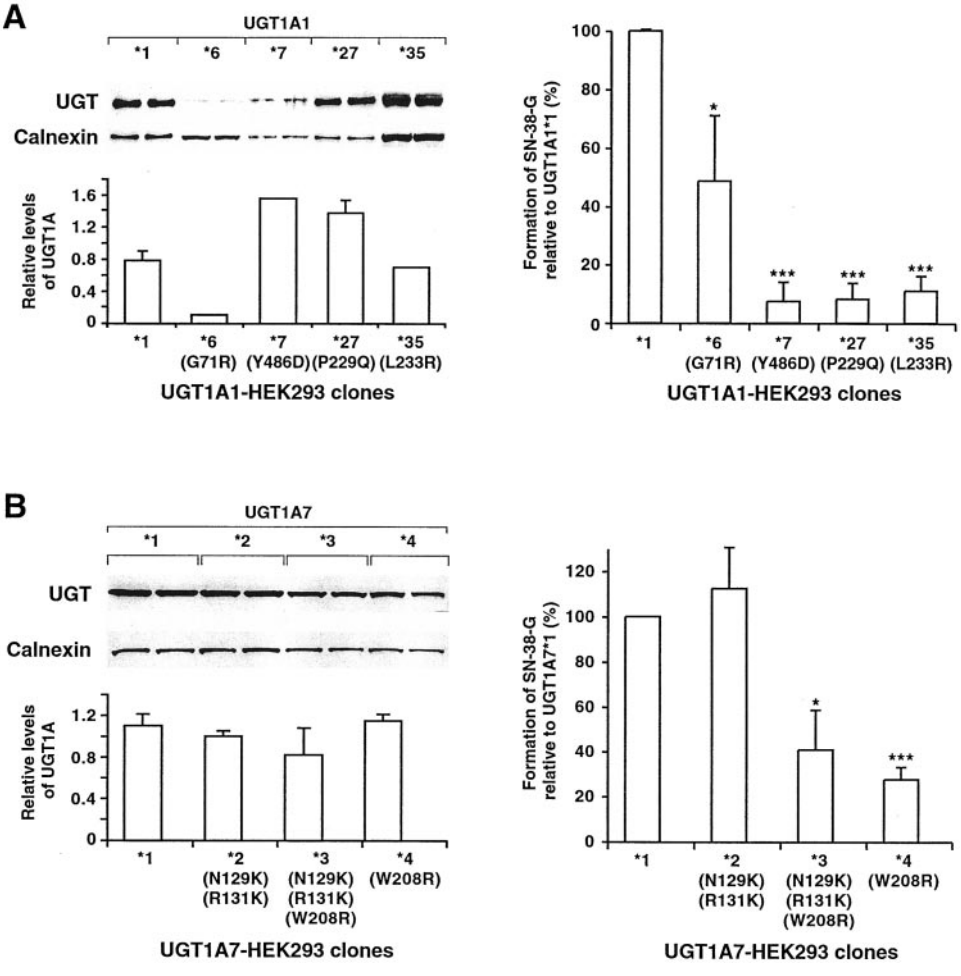

Fig. 5. Relative expression of the UGT1A1 and UGT1A7 alleles and catalytic activities for SN-38. Membrane fractions prepared from one selected clone (run in duplicate) for each UGT variant HEK cells expressing the UGT1A1 (A) and UGT1A7 (B) variants (20 μg of protein loaded in each lane) were subjected to SDS-PAGE, transferred onto nitrocellulose and probed with antisera raised against human UGT1A. The blot was subsequently probed with a polyclonal anti-calnexin antibody. Relative levels of UGT1A determined by semiquantitative densitometric analysis of the ECL image are shown on left. Ratios between UGT and calnexin signals were calculated for each UGT cell lines. The expression of *UGT1A7*1* was arbitrarily designated as the basal UGT1A expression level and all UGT compared with *UGT1A7*1*. Glucuronidating activities for variant UGT proteins were determined as described under *Experimental Procedures*. The activity of variants UGT is compared with *UGT*1* alleles and data are presented in percentage of activity relative to allele *1. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.002$ vs *1 alleles.

tive importance of UGT1A9 compared with UGT1A1 in the *in vivo* metabolism of SN-38 still remains to be determined.

Wide interindividual variation have been observed in the metabolism of irinotecan (Gupta et al., 1994; Kudoh et al., 1995; Lokiec et al., 1996; Iyer et al., 1998). By measuring the variability of SN-38 glucuronide formation, we confirmed a high interindividual glucuronidation variability and suggest that the capacity of hepatic SN-38 glucuronidation is highly dependent on the level of UGT1A expression in most liver samples. This could be explained by a number of factors, including exposure to therapeutic drugs, diet, environmental factors, and diseases, but also by the presence of genetic variations in the regulatory regions of SN-38 glucuronidating UGT enzymes. In fact, interindividual differences in pharmacokinetics of SN-38 and SN-38-G were associated with UGT1A1 promoter genotypes (Ando et al., 1998). Correlation between low and high UGT1A1 promoter glucuronidating alleles and rate of glucuronidation of SN-38 has been established (Iyer et al., 1999). The hypothesis was then raised that UGT1A1 promoter polymorphism could help predict irinotecan-associated toxicity (Ando et al., 2000). We assessed the effect of additional polymorphisms present in the coding region of UGT1A1. These genetic variants were previously reported either as common polymorphism in the Japanese population (*UGT1A1*6* $G^{71}R$), variant of UGT1A1 allele associated with Gilbert's syndrome (*UGT1A1*27* $P^{229}Q$) and rare mutations (*UGT1A1*7* $Y^{486}D$) associated with severe chronic nonhemolytic unconjugated hyperbilirubinemia or found in a patient with breast cancer (*UGT1A1*35* $L^{233}R$; C. Guillemette and D. E. Housman, unpublished observations). Biochemical characterization of these selected UGT1A1 variants demonstrated a dramatic negative effect on the formation of SN-38-G explained by lower rates. As expected, an important decrease in SN-38-G formation was observed for the *UGT1A1*7* variant, which was previously shown to dramatically reduce bilirubin glucuronidation (Aono et al., 1993). A probable negative impact of the *UGT1A1*6*, **27*, and **35* variants in the glucuronidation of SN-38 in vivo is suggested due to the dramatic decrease in SN-38-G formation observed in vitro, ranging from 51 to 93%. Based on the functional data, heterozygous for the *UGT1A1*27* and **35* alleles would present half the SN-38 glucuronidating activity of homozygous individuals for the *UGT1A1*1* allele and may be associated with toxicity induced by irinotecan therapy. In fact, the recent study of Ando et al. (2000) showed that all patients heterozygous for the *UGT1A1*27* allele experienced severe toxicity (Ando et al., 2000). However, no patients were found to be homozygous for the *UGT1A1*27* allele and the authors explained that these patients were automatically excluded from the analysis because they would have die of fatal toxicity. Given the rare frequency of the *UGT1A1*35* allele initially discovered in an early onset breast cancer patient (C. Guillemette and D. E. Housman, unpublished observations), its impact in the general population may be limited. In contrast, Akaba et al. (1999) reported that the *UGT1A1*6* $G^{71}R$ allele was relatively frequent in the Japanese population with 30% heterozygous individuals. Furthermore, this allele was associated with a significant decreased in bilirubin glucuronidation (Akaba et al., 1998). Yet given the high frequency of the $G^{71}R$ variation (*UGT1A1*6*) in the Japanese population and the significant negative impact observed herein on SN-38-G formation, its potential association with irinotecan-induced toxicity was shown to be limited (Ando et al., 2000).

The accumulation of SN-38 in the gastrointestinal tract was proposed as the most probable explanation for intestinal toxicity. Deconjugation of SN-38-G to form SN-38 by β-glucuronidases in the intestinal microflora was suggested as a main contributing factor (Araki et al., 1993). Therefore, the conjugation of SN-38 locally in the intestine would play a protective role in reconjugating the SN-38 avoiding accumulation. The extra-hepatic UGT1A7 presented the highest catalytic activity toward SN-38 of all UGT enzymes. Common UGT1A7 functional variants *UGT1A7*3* and *UGT1A7*4* were shown to negatively impact SN-38-G formation; however, levels of UGT1A7 transcript were undetectable in the intestine, the colon, or in the liver (Tukey and Strassburg, 2000). Yet given the high frequency of the UGT1A7 variant alleles in the population and their negative impact on SN-38 glucuronidation, their potential association with severe toxicity induced by irinotecan is unlikely. In contrast, UGT1A1 and UGT1A9 transcripts were detected in the GI tract (Tukey and Strassburg, 2000). Accordingly, these two UGT would represent potential key enzymes involved in the protection of GI toxicity induced by irinotecan treatment.

In vitro metabolic studies also suggest the possible involvement of UGT1A6, UGT1A8, and UGT1A10 although much lower intrinsic clearances were observed compared with UGT1A1, UGT1A7, and UGT1A9. To date, although no polymorphic UGT1A8, UGT1A9, or UGT1A10 has been described, one study reports the absence of functional polymorphism in the UGT1A9 first exon of 129 patients with hepatocellular carcinoma (Vogel et al., 2001), whereas a functional UGT1A6 variant was reported (Ciotti et al., 1997). The study of the catalytic activity of the polymorphic *UGT1A6*2* variant allele ($A^{181}S^{184}$) suggested an altered formation of SN-38-G compared with *UGT1A6*1* allele (data not shown), whereas intrinsic clearance would predict limited impact of this allele on the formation of SN-38-G in vivo. Accordingly, a previous report showed a poor correlation between *para*-nitrophenol, a substrate of UGT1A6, and SN-38 glucuronidation (Iyer et al., 1998). Court et al. (2001) estimated a relatively minor contribution of UGT1A6 and UGT1A1 compared with UGT1A9 in the hepatic glucuronidation of acetaminophen; however, phenotyping of acetaminophen in cancer patients was proved to be a poor predictor of SN-38 glucuronidation (Gupta et al., 1997). Consistent with these observations, our results regarding UGT1A6 kinetic characteristics would suggest a minor role of this hepatic UGT1A enzyme in the formation of SN-38 glucuronide in vivo compared with the hepatic UGT1A1 and UGT1A9.

In conclusion, little information has been available regarding the existence and the clinical relevance of genetic polymorphisms among members of the *UGT* gene family, despite increasing evidence that supports a protective role of these enzymes in irinotecan-associated toxicity (Ando et al., 2000). Phenotypic characterization of polymorphic UGT isoenzymes relevant to SN-38 glucuronidation revealed that common genetic variants of *UGT1A1* and *UGT1A7* genes result in a significantly decrease in SN-38 glucuronidating capacity. These results support that cancer patients presenting *UGT1A* genotypes investigated here, either alone or combined to the UGT1A1*28 promoter genotype, could present significant impaired SN-38 glucuronidating capacity. These

patients may present altered response to irinotecan therapy and be at increase risk for adverse reactions. In addition, although the relative importance of UGT1A9 in the in vivo SN-38 glucuronidation compared with UGT1A1 remains to be demonstrated, data suggests that molecular determinants of irinotecan response may include variants of the hepatic UGT1A9 isoenzyme. Our preliminary results in resequencing *UGT1A9* gene led to the identification of nonsynonymous single nucleotide polymorphisms in the UGT1A9 (data not shown). These results warrant additional investigation to characterize the function of newly identified UGT1A9 polymorphisms and reveal their clinical importance. The expression pattern of UGT1A1 and UGT1A9 in the liver and the gastrointestinal tract, where the SN-38 toxicity takes place (Tukey and Strassburg, 2000), suggests that an altered activity or expression of these metabolic enzymes could be associated with an increase toxicity to irinotecan therapy. Therefore, the coexistence of *UGT1A* polymorphisms may greatly affect the individual response and patient susceptibility to irinotecan toxicity. The large variation in the UGT activity being related to the patient UGT genotype, genotyping of *UGT1A* could help adjust the dosage of a patient based on genetic status to prevent severe toxicity of irinotecan.

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