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Glucuronidation of 7-Ethyl-10-hydroxycamptothecin (SN-38) by the Human UDP-Glucuronosyltransferases Encoded at the *UGT1* Locus

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7-Ethyl-10-hydroxycamptothecin (SN-38) is a very promising anticancer drug used for the treatment of metastatic colonrectal cancer. SN-38 is the active metabolite of irinotecan, a semisynthetic anticancer drug derived from 20(S)camptothecin. In this study, we examined the potential for each of the *UGT1*-encoded isoforms (UGT1A1 and UGT1A3 through UGT1A10) to glucuronidate SN-38. The amount of specific protein for each isoform was determined by Western blot analysis. Although UGT1A1 was previously shown to metabolize this drug, the results of this study show that UGT1A7 glucuronidates this chemical at a 9- to 21-fold higher level at pH 6.4 and pH 7.6, respectively, than that by UGT1A1. The activity of UGT1A7 is from 8.4- to 19-fold higher at pH 6.4 and 12- to 40-fold higher at pH 7.6 than that by the other 7 *UGT1* encoded isoforms. UGT1A7 glucuronidates SN-38 with an apparent Km of 5 μM. Hence, the distribution of this isoform in the gastrointestinal tract has the potential to impact the effectiveness of this chemotherapeutic agent.

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin), also known as CPT-11, is a semisynthetic derivative of 20(S)camptothecin with a promising anticancer activity in metastatic colonrectal cancer (1). Irinotecan is biotransformed by serum and tissue carboxylesterases to a much more potent metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin) (2), which has 100- to 1,000-fold higher antitumor activity than irinotecan. Hence, irinotecan can be assumed to be a prodrug of SN-38.

SN-38 undergoes significant glucuronidation, biliary excretion, and enterohepatic circulation (3). In fact, it is glucuronidated to 10-O-glucuronyl-SN-38 and excreted into the bile and urine. Furthermore, SN-38 can be deconjugated to form SN-38 by intestinal bacterial β-glucuronidase (4). The dose-dependent diarrhea is due to the accumulation of SN-38 in the intestine (5). It is suggested that inhibition of β-glucuronidase activity in the intestinal microflora with antibiotic therapy would ameliorate the diarrhea caused by SN-38 and reduce the intestinal damage and toxicity.

In any event, carboxylesterase is not the only pathway for the detoxification of irinotecan. In fact, the cythocrome P450 A3 enzyme can metabolize irinotecan to 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC), which has much less antitumor activity than SN-38 (6, 7).

The purpose of this study was to determine which of the *UGT1*-encoded UDP-glucuronosyltransferases (transferase) is most responsible for SN-38 glucuronidation. Specific protein amounts were established by Western blot analysis by using an antibody to the common-end of all proteins encoded at this locus. Protein extract of transiently transfected COS-1 cells with either pUGT1A1 or UGT1A3 through pUGT1A10 was used in each case to assess the level of glucuronide formed with SN-38.

MATERIALS AND METHODS

Materials. The sources of the list of reagents used in this study have been previously identified (8, 9, 10). SN-38 was from Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD).

Isolation or synthesis of the UDP-glucuronosyltransferase cDNA expression units and transfection into COS-1 cells. The isolation and construction of UGT1A1, UGT1A4 and UGT1A6 have already been described (9, 11). UGT1A9 was isolated from a λZAP cDNA library which was custom synthesized by Invitrogen (Carlsbad, CA) using mRNA from a specimen of a normal human liver prepared for transplant surgery. UGT1A9 was cloned in pSVL as follows: both the UGT1A9 cDNA based-pSK+ unit and the UGT1A1 cDNA-based pSVL were digested with *XhoI/BstEII* to generate a 1140 bp and 1099 bp fragment, respectively. The *XhoI/BstEII* UGT1A9 fragment

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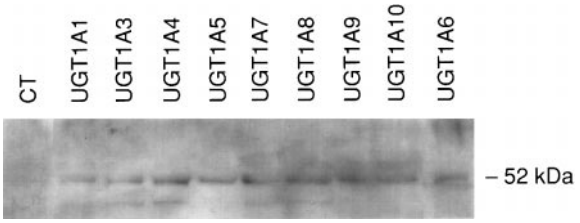

FIG. 1. Western blot analysis of UGT1A1 and UGT1A7 through UGT1A10 expressed in COS-1 cells. A total of 25 μg microsomal protein was electrophoresed on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a UGT1 common end rabbit antibody as described under Materials and Methods. Protein samples are: Lane 1, mock transfected cells; lanes 2 to 10, cells transfected with each specific UGT1 isoform as labeled.

replaced the *XhoI/BsTEII* UGT1A1 fragment in the *XhoI/BstEII*-digested UGT1A1 cDNA based-pSVL via a ligation reaction. The *XhoI/BstEII* digested pSVL contained the 1099-2100 bp of the common 3′ end of the cDNAs. The details for the construction of the cDNA expression units for UGT1A3, UGT1A5, UGT1A7, UGT1A8, and UGT1A10 are described elsewhere (M. Ciotti/N. Basu/M. S. Hwang, J. W. Cho, A. F. McDonagh, and I. S. Owens, manuscript in preparation). The plasmids were transfected into COS-1 cells using diethylaminoethyldextran followed by chloroquine treatment (10). The transfected cells were harvested after 72 hr and stored at −80°C until used in the Western blot analysis and the enzyme assays.

Western blot analysis of the UGT1-encoded proteins. Total protein (25 μg) extract of each transfected cell was electrophoresed on a 15% SDS-polyacrylamide gel supplied by BioRad (Hercules, CA). The separated proteins were electrotransferred to nitrocellulose membrane and probed with a rabbit IgG polyclonal antibody made against the common carboxyl terminus of all *UGT1*-encoded isoforms (M. Ciotti/N. Basu/M. S. Hwang, J. W. Cho, A. F. McDonagh, and I. S. Owens, manuscript in preparation). A second goat anti-rabbit IgG HRP conjugate was added 1 hr later and the proteins were visualized according to the ECL Western blotting protocol (Amersham, Arlington Heights, IL). The relative intensity of the protein bands was assessed by scanning and quantifying by using the Adobe photoshop 3.0 software.

Assay for glucuronidation using protein extract of transfected cells. The SN38-glucuronidating activity was measured for each of the proteins encoded by either UGT1A1-, UGT1A3-, UGT1A4-, UGT1A5-, UGT1A6-, UGT1A7-, UGT1A8-, UGT1A9-, or UGT1A10-cDNA following expression in COS-1 cells. Product was measured by thin layer cromatography (TLC) as previously described (10). Briefly, incubations contained 300 μg detergent-treated protein and 200 μM SN-38; other reactants were 1.41 mM [^{14}C]UDP-glucuronic acid (1.4 mCi/mmol), 5mM $MgCl_2$, 16.6 mM saccharic acid 1,4-lactone, and either 33 mM triethanolamine, pH 7.6 or 20 mM sodium phosphate, pH 6.4 in a total volume of 100 μl. The reactions were carried out at room temperature for 16 hr and stopped with 200 μl 95% ethanol. The glucuronides were analyzed by TLC chromatography and scanned on the Ambis Radioanalytical Imaging System II for quantification of product as previously described (10). SN-38 was also tested with mock transfected COS-1 cells which exhibited no detectable activity above background. Based on the results of the Western blot analysis, the product generated by UGT1A4 was corrected for having 10% more specific protein than all other cDNA-based expression systems.

Km determination of UGT1A7 towards SN-38. In order to determine the Michaelis-Menten costant of UGT1A7 toward SN-38, 300 μg of cell homogenate from COS-1 cells transfected with pUGT1A7 were incubated with different concentrations of SN-38 ranging from 0.5 to 200 μM for 2 hr at 37°C.

Sensitivity of product(s) to β-glucuronidase. To demonstrate that the product(s) generated by UGT1A7 at both pH 6.4 and pH 7.6 were β-glucuronides, it was necessary to treat the sample with β-glucuronidase. The amount of β-glucuronidase required to degrade the β-glucuronides was 12.5 units upon adjusting the reaction to pH 6.4.

RESULTS AND DISCUSSION

Western Blot Analysis of Transfected COS-1 Cells

The Western blot analysis of cell lysate revealed the presence of specific protein for each of the expression units as shown in Fig. 1. The 15% SDS-polyacrylamide gel revealed a discrete protein band expressed by each cDNA; however differences in the molecular weight for some of the 9 different proteins due to differences in the number of glycosylation sites are not evident. The results show, however, that each protein was expressed to the same extent. The product generated by

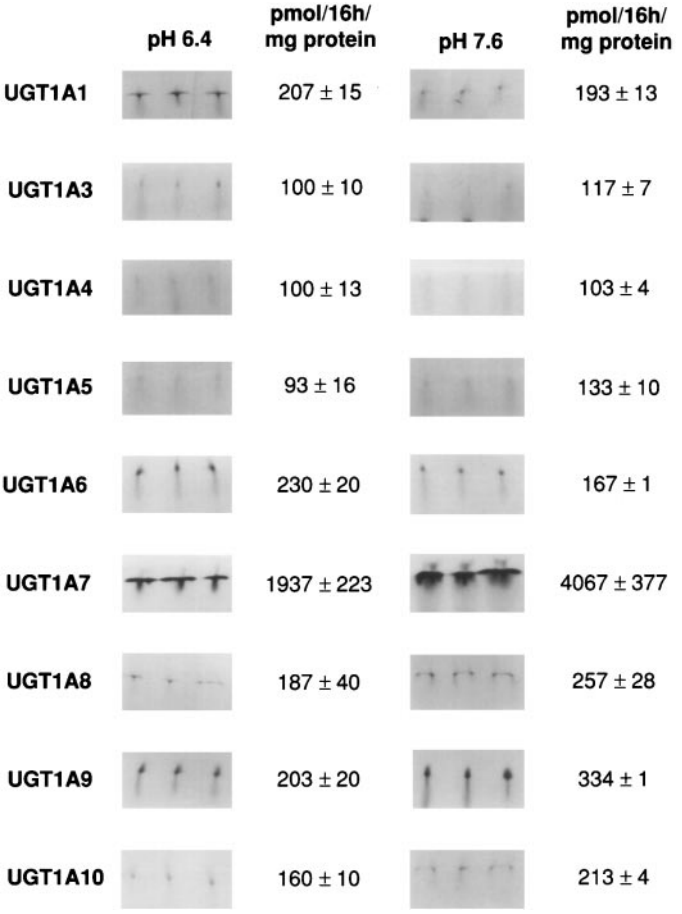

FIG. 2. Analysis of SN-38 glucuronide(s) generated by the UGT1 isoforms at pH 6.4 and pH 7.6. COS-1 cells were transfected with each specific UGT1 isoform and harvested after 72 hr as described under Materials and Methods. Equivalent amounts of specific protein were used to generate SN-38 glucuronide(s) as described under Materials and Methods using 1.4 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol) with 200 μM SN-38.

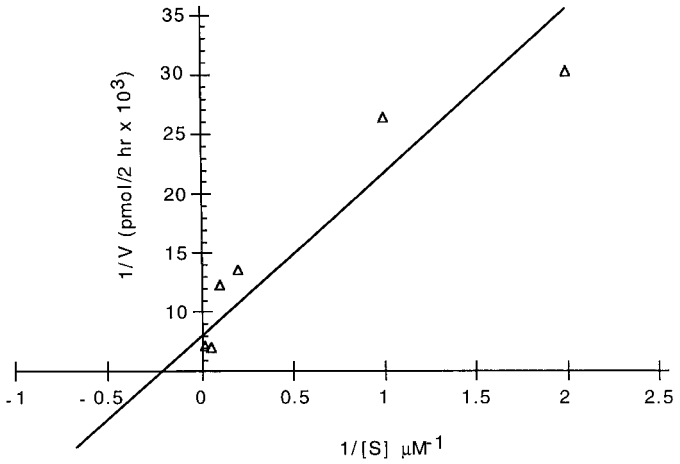

FIG. 3. Lineweaver-Burk plot of SN-38 concentrations versus reaction velocity of glucuronidation using protein from pUGT1A7-transfected COS-1 cells. The effect of SN-38 concentration on velocity was analyzed using a range of SN-38 concentrations with 1.4 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol), and 300 μg of cell homogenate protein at pH 7.6 as described under Materials and Methods; incubations were carried out at 37°C for 2 hr.

UGT1A4 was corrected for 10% more specific protein than each of the other transferases. It can be seen that no comparable protein was detected in the mock-transfected cells.

Glucuronidation of SN -38 by Protein Extract of Transfected COS-1 Cells

Glucuronide formation by the UDP-glucuronosyltransferase system is one of the major pathways in mammalians for the inactivation of endobiotics and xenobiotics generally leading to excretable products. In particular, this is a primary pathway involved in the clearance of SN-38. Hence, we inquired as to the most active SN-38-glucuronidating isoform encoded at the *UGT1* locus. It can be seen in Fig. 2 that UGT1A7 glucuronidated this drug with a ratio of 2:1 with respect to pH 7.6: pH 6.4 as shown by 4067 ± 377: 1937 ± 223 pmol SN-38 β-glucuronide/16 hr using 300 μg of total cell protein. The other *UGT1*-encoded isoforms formed 93–230 pmol glucuonide at pH 6.4 to no more than 103 to 334 pmol at pH 7.6. Thus, the UGT1A7 conjugates this chemical with a ratio from 8.4- to 21-fold higher at pH 6.4 than either of the other isoforms, and it glucuronidates 12- to 40-fold better at pH 7.6 than any of the other isoforms. Although the quantities of product formed by the other isoforms were particularly low, it can be seen that UGT1A1 and UGT1A6 glucuronidated with a slight preference for pH 6.4-, whereas all other isoforms glucuronidated SN-38 with a slight preference for pH 7.6. Recently, it was reported that UGT1A1 (12) glucuronidates this anticancer drug. Based on the fact that UGT1A7 glucuronidates SN-38 to a far greater level than each of the other *UGT1*-encoded isoforms and that this isoform is present at relative low levels in the colon according to Northern blot analysis (I. S. Owens, Glucuronidation Workshop, The University of Iowa, Iowa City, IA, May 1996; M. Ciotti/N. Basu/M. S. Hwang, J. W. Cho, A. F. McDonagh, and I. S. Owens, manuscript in preparation), it may be that the low level of UGT1A7 in this tissue is the basis for the failure to efficiently re-glucuronidate bacterially hydrolyzed SN-38-β-glucuronide. RT- PCR analysis of RNA found the UGT1A7 enzyme in gastrointestinal tissue (13). UGT1A9, present at much higher levels in the colon than UGT1A1 (M. Ciotti/N. Basu/M. S. Hwang, J. W. Cho, A. F. McDonagh, and I. S. Owens, manuscript in preparation), should have a greater impact on the glucuronidation of SN-38 than UGT1A1 with both having equivalently low activity.

Also it is possible that SN-38 is not efficiently reabsorbed by the colon to undergo glucuronidation, and that its damages is directed from the lumen of the colon.

The product formed at both pH values is shown to be completely sensitive to β-glucuronidase treatment (Fig. 3) confirming that the product(s) generated by UGT1A7 is a glucuronide(s).

Apparent Km of UGT1A7 Toward SN-38

Since only UGT1A7 glucuronidated SN-38 at a level which allowed the determination of a Km value, we show in Fig. 4 an apparent Km value of 5 μM at pH 7.6 and a Vmax of 0.17 pmol/2hr/mg protein. Based on the dosages of irinotecan administered (125mg/m^2 every 3 weeks and twice this dosage in Europe) to patients and its hydrolysis by both a low and a high Km carboxylesterase, SN-38 concentrations easily approach millmolar levels in the liver and intestinal cells (14). Hence, the Km for its glucuronidation by UGT1A7 is easily

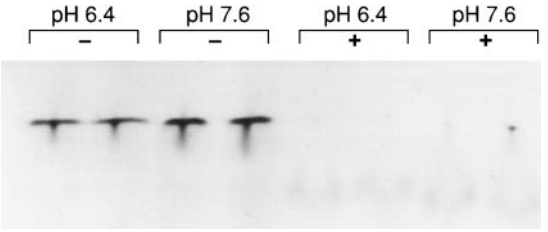

FIG. 4. Sensitivity of SN-38-β-glucuronide(s) to β-glucuronidase. The protein extract (300 μg) of pUGT1A7-transfected COS-1 cells in combination with 1.4 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol) and 200 μM SN-38 was used to generate β-glucuronides of SN-38 at pH 6.4 or pH 7.6 during a 16 hr incubation at 25°C. Half of each set of reactions was untreated (−) or treated (+) with β-glucuronidase. *E. coli* β-glucuronidase was added directly to the pH 6.4 mixtures, while the pH 7.6 reaction mixtures were brought to pH 7.0 with 40 mM sodium phosphate (pH 7.0) before adding the enzyme. The reaction mixtures were treated with 12.5 U of the enzyme for 3 hr at 37°C, and then analyzed on TLC plates as decribed under Materials and Methods.

met in certain tissues following the administration of the prodrug. Although our characterization (M. Ciotti/N. Basu/M. S. Hwang, J. W. Cho, A. F. McDonagh, and I. S. Owens, manuscript in preparation) shows that UGT1A7 is a very robust enzyme which is able to glucuronidate chemicals at high substrate levels, unlike inhibition by high substrate for some of the other *UGT1*-encoded isozymes, it is unlikely that the low level of this isozyme in the colon can handle the extremely high concentrations of SN-38 predicted to form as a consequence of SN-38-*β*-glucuronide hydrolysis by colon microflora given the high dosages involved. Hence, the unfortunate high concentration of SN-38 required for a chemotherapeutic effect, in conjunction with the low level of the UGT1A7 in colon, is likely the basis for its toxicity and the resulting diarrhea. Based on this study we would not predict that any of the other *UGT1*-encoded isoforms will likely impact very much the glucuronidation of this drug in the gastrointestinal tract.

Our finding that UGT1A7 is widespread in the body (data not shown), mostly at low levels, might lead to a better understanding of the basis of the clearance or lack of clearance of this drug. Further, UGT1A7 is a potential target enzyme for studying differences in effects among different patient populations. Population pharmacokinetic studies of CPT-11, SN-38, and SN-38 glucuronide in Caucasian and Japanese individuals (15, 16) suggest ethnic differences in the metabolic profile of CPT-11. For these reasons, it would be very important to search for genetic polymorphisms and/or mutations in the UGT1A7 isoform.

In fact, it is known that there is a genetic predisposition to develop gastrointestinal toxicity and neutropenia after irinotecan treatment (17) in patients with Gilbert's syndrome. Since UGT1A7 is not likely to be defective in individuals with Gilbert's syndrome, except for those patients harboring a mutation in one of the common exons (2–4), it would be worth distinguishing the site of mutations in affected individuals to understand whether this enzyme is pivotal in the toxicity caused by this drug. The finding that UGT1A7 is able to glucuronidate SN-38, the active metabolite of irinotecan (CPT-11), at a very high level shed new light on the molecular mechanism underlying the glucuronidation of SN-38, a metabolite of irinotecan.

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