**ABSTRACT:**

The metabolic pathway of CPT-11 is the bipiperidine side chain oxidation by human UGT1A1 variants, G71R, P229Q, and Y486D.

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**GLUCURONIDATION OF 7-ETHYL-10-HYDROXYCAMPOTHECIN (SN-38), AN ACTIVE METABOLITE OF IRINOTECAN (CPT-11), BY HUMAN UGT1A1 VARIANTS, G71R, P229Q, AND Y486D**

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**ABSTRACT:**

7-Ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of antitumor agent irinotecan (CPT-11), is conjugated and detoxified to SN-38-glucuronide by UDP-glucuronosyltransferase (UGT) 1A1. Genetic polymorphisms in UGT1A1 are thought to contribute to severe diarrhea and/or leukopenia caused by CPT-11. In this regard, it has been reported that polymorphisms in the promoter region could affect the CPT-11 pharmacokinetics and interindividual variation of toxicity. However, little information is available on the influence of UGT1A1 polymorphisms in the coding region on the SN-38 glucuronidation activity. In the present study, wild-type (WT) and three variant (G71R, P229Q, and Y486D) CDAs of human UGT1A1s were transiently expressed in COS-1 cells, and the kinetic parameters of these UGT1A1s were determined for SN-38 glucuronidation. A partially reduced UGT1A1 protein expression was observed in COS-1 cells for G71R and Y486D. WT UGT1A1 catalyzed SN-38 glucuronidation with an apparent $K_m$ value of 11.5 $\mu$M, whereas those of G71R, P229Q, and Y486D were 14.0, 18.0, and 63.5 $\mu$M, respectively. The SN-38 glucuronidation efficiency ratio ($V_{\max}/K_m$) normalized for the level of expression was 1.4, 0.68 (47% of WT), 0.73 (52%), and 0.07 (5%) $\mu$mol/min/mg of protein for WT, G71R, P229Q, and Y486D, respectively. Thus, the SN-38 glucuronidation activity of Y486D was drastically reduced, whereas the reduction in the G71R and P229Q activities was fractional. The decreased SN-38 glucuronidation efficiency ratio of G71R and P229Q could be critical in combination with other polymorphisms in the UGT1A1 gene.
Denaturation at 95°C for 1 min, followed by 30 cycles of incubations at 95°C each of forward and reverse primers. The cycling parameters were as follows:

- Forward primer: 5’-CCTCGTTGTAACATGAGAGAGACTTTTACCTTTGGAAG-3’
- Reverse primer: 5’-CTTCAAGGTGTTAAATGCTCCTTCAAGGACGTTTGTATGCAACCCTTGCCTC-3’

The 1000 ng mixture contained 5 units of TaKaRa DNA polymerase (Takara, Kyoto, Japan), 1.5 mM MgCl₂, 50 μM deoxynucleoside-5’-triphosphate and 0.2 μM each of forward and reverse primers. The cycling parameters were as follows:

- Denaturation at 95°C for 1 min, followed by 30 cycles of incubations at 95°C for 30 s and at 67°C for 2 min, and termination by 10-min extension at 72°C.

The PCR products were cloned into pCR-2.1 by the TA cloning procedure (Invitrogen, Carlsbad, CA). The resulting plasmid (pCR-UGT1A1/WT), containing the correct insert in a reverse orientation, was then excised with NotI and BamHI, and the expression plasmids were transfected into COS-1 cells, pcDNA-UGT1A1/P229Q, and pcDNA-UGT1A1/Y486D, respectively.

**Materials and Methods**

**Materials.** SN-38 (Lot 970507R) and SN-38G (Lot 970507R) were kindly supplied by Yakult Honsha Co. Ltd. (Tokyo, Japan). Human adult normal liver cDNA was purchased from BioChain Institute Inc. (Hayward, CA). COS-1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan).

**Construction of Plasmids.** The cDNA of wild-type UGT1A1 was amplified by PCR from human adult normal liver cDNA using the forward primer, 5’-CAAGCCGCCCAGGCTGCG-3’ and the reverse primer, 5’-CTTTATCCCACCTTCTTCAG-3’ The PCR products were cloned into pCR2.1 by the TA cloning procedure (Invitrogen, Carlsbad, CA) and sequenced in both the forward and reverse directions on an Applied Biosystems 3700 sequencer (Applied Biosystems, Foster City, CA). The resulting plasmids were transfected using a LipofectAMINE 2000 complex (Invitrogen, Carlsbad, CA). Visualization was achieved with horseradish peroxidase conjugated donkey anti-rabbit IgG (1:2500) and enhanced chemiluminescence-plus (Biorad, Hercules, CA).

**Expression of Wild-Type and Mutant UGT1A1s in COS-1 Cells.** COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The day before transfection, COS-1 cells were plated at 100,000 cells/dish at a density of 5.5 x 10⁵ cells/cm². On the following day, the culture medium was replaced with 8 ml of Opti-MEM (Invitrogen), and the expression plasmids were transfected using a LipofectAMINE 2000 reagent (Invitrogen). The diluted DNA (14 μg in 810 μl of Opti-MEM) and the diluted LipofectAMINE 2000 reagent (16 μl in 810 μl of Opti-MEM) were combined and incubated for 20 min at room temperature. The resulting DNA-LipofectAMINE 2000 complex was added directly to each dish.

**Western Blotting.** Twenty micrograms of the membrane fraction protein from COS-1 cells were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membrane. Immunoblotting of each UGT1A1 protein was performed using a rabbit anti-human UGT1A1 (diluted at 1:2500; BD Gentest, Woburn, MA). To verify that the samples were evenly loaded, the blot was subsequently treated with the stripping buffer and reprobed with a polyclonal anti-calnexin antibody (diluted at 1:10000; Stressgen Biotechnologies Inc., San Diego, CA). Visualization was achieved with horseradish peroxidase conjugated donkey anti-rabbit IgG (1:2500) and enhanced chemiluminescence-plus reagents (Amersham Biosciences Inc., Piscataway, NJ). The band intensities were quantitated using baculovirus cell lysate containing recombinant

2000), the effect on the SN-38 glucuronidation activities of these polymorphic variations has not been reported except for TA polymorphism in the promoter.

In the present study, wild-type and three variant UGT1A1s (G71R, P229Q, Y486D) were transiently expressed in COS-1 cells, and their kinetic parameters for SN-38 glucuronidation were determined by the high-performance liquid chromatography method with fluorescence detection (Hanioka et al., 2001a). Among these variants, G71R is the most common in the East Asian population (allele frequencies of 13–23%), followed by P229Q (2.8%) (Akaba et al., 1998; Huang et al., 2000). Y486D is a rather rare variant found in Japanese patients with Crigler-Najjar syndrome type II (Yamamoto et al., 1998a). Recently, Gagné et al. (2002) have reported the SN-38 glucuronidation by the same variant UGT1A1s, but detailed kinetic analysis was not included for the variants. The data presented here would provide the additional information on the clinical significance of UGT1A1 polymorphism.
UGT1A1 protein (BD Gentest) as a standard, under conditions in which band densities and protein levels of standard UGT1A1 were linearly related.

**Real-Time Reverse Transcription (RT)-PCR.** Total cellular RNA was isolated from the COS-1 cells using the RNaseasy Mini kit with the addition of RNase-free DNase to prevent DNA contamination of samples (Qiagen, Tokyo, Japan). First-strand cDNA was prepared from 200 ng of total cellular RNA with oligo(dT) primer and MultiScribe reverse transcriptase (Applied Biosystems). RT-PCR assays based on SYBR Green were performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The primers used to amplify UGT1A1 were 5′-TAGTTGCTCTGACCTGAGGC-3′ (forward) and 5′-TCTTTCAACTCCTCCCTTTGG-3′ (reverse). The 50-µl reaction mixture contained 25 µl of SYBR Green PCR Master Mix (Applied Biosystems), 2 µl of cDNA corresponding to 4 ng of reverse-transcribed total RNA and 0.1 µM each of forward and reverse primers. Thermocycling conditions were as for standard TaqMan protocol, 10 min denaturation at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. In each reaction, the size of the PCR product (100 base pairs) was confirmed by agarose gel electrophoresis. The samples without reverse transcriptase were routinely included in the RT-PCR reactions to measure the possible interference of the contaminated DNA, which was usually less than 5% of the RNA-derived amplification. Transcripts of β-actin were quantified as internal controls using TaqMan β-actin control reagents, and each sample was normalized on the basis of its β-actin content.

**Enzyme Assay.** The glucuronidation activities of wild-type and mutant UGT1A1s were assayed as described previously (Hanioka et al., 2001a). Briefly, the incubation mixtures contained 50 mM Tris–HCl buffer, pH 7.4, SN-38 (2.5–150 µM), the membrane fraction of COS-1 cells (100 µg of protein), 10 mM MgCl₂, and 5 mM UDP-glucuronic acid. After preincubation at 37°C for 1 min, the reaction was started by the addition of UDP-glucuronic acid. The mixture was incubated at 37°C for 80 min, and the reaction was terminated with 100 µl of 10% (w/v) HClO₄. After centrifugation at 12,000g for 10 min at 4°C, the clear supernatant was filtered using a 0.45 µm polytetrafluoroethylene membrane filter, and analyzed by high-performance liquid chromatography. The limit of quantitation for SN-38G was 2 pmol/ml, which corresponded to the enzyme activity of 0.1 pmol/min/mg of protein.

**Data Analysis.** Results were analyzed using one-way analysis of variance for variance followed by Dunnett’s test as post hoc test. Kinetic parameters were calculated with Prism 3.0 (Graph Pad Software, Inc., San Diego, CA), using nonlinear regression of Michaelis-Menten equation. Kinetic parameters shown are the results from three independent preparations.

**Results**

**Expression of Wild-Type and Mutant UGT1A1s in COS-1 Cells.** The mutation (211G→A, G71R; 686C→G, P229Q; 1456T→G, Y486D) was introduced to pCR-UGT1A1/WT using the PCR-based site-directed mutagenesis. The wild-type (WT) and mutant cDNAs were subcloned into mammalian expression plasmid pcDNA3.1(−) and transiently transfected into COS-1 cells. The relative expression levels of each UGT1A1 protein in the membrane fraction were determined by Western blotting with a polyclonal anti-human UGT1A antibody (Fig. 2). The protein expression levels were found to vary among the four UGT1A1s, from a low of approximately 40% of WT in G71R-expressing cells to a high of 120% in the P229Q-expressing cells. This difference in relative levels of expression was reproducible in three independent transfection experiments, and the reduced expression of G71R and Y486D proteins compared with that of WT was statistically significant (by one-way analysis of variance and Dunnett’s test, p < 0.05).

To further address the mechanisms involved in this phenomenon, the UGT1A1 mRNA expression level in the transfected COS-1 cells was measured by real-time RT-PCR using SYBR Green reagents. As shown in Fig. 3, no significant difference in the level of mRNA was detected among the COS-1 cells transfected with the expression plasmids carrying each UGT1A1 cDNA. This suggests that the observed difference in UGT1A1s was not due to the different transcription or transfection efficiency of the plasmid constructs.

**SN-38 Glucuronidation by Wild-Type and Mutant UGT1A1s.** The apparent kinetic parameters (Vmax, Vmax/Km) were determined for SN-38 glucuronidation by the wild-type and mutant UGT1A1s expressed in COS-1 cells. The assays were performed by using 11 substrate concentrations between 2.5 and 150 µM. The representative nonlinear regression curves of the Michaelis-Menten kinetics are shown in Fig. 4. The Vmax values were normalized to that of WT. The results were expressed as the mean ± S.E.M. from three-independent preparations.

**Table 1** summarizes the apparent kinetic parameters for SN-38...
SN-38 glucuronidation by expressed UGT1A1s was assayed in the presence of the membrane fractions (100 μg) at the substrate concentration range between 2.5 and 150 μM. The solid line indicates fitting of data to the Michaelis-Menten equation by the nonlinear regression.

SN-38G, plays a significant role in protecting against these side effects of CPT-11 (Iyer et al., 1998), whereas hepatic UGT1A9 (Hanioka et al., 2001b) and extrahepatic UGT1A7 (Ciotti et al., 1999b) could also catalyze this conjugation reaction. UGT1A1 is responsible for the glucuronidation of an endogenous substrate bilirubin, and some of the polymorphisms in UGT1A1 gene are associated with hyperbilirubinemia, such as Crigler-Najjar syndrome type I and type II, and Gilbert’s syndrome. In the present study, we investigated the SN-38 glucuronidation activities of three variant UGT1A1s, G71R, P229Q, and Y486D, expressed in COS-1 cells. G71R (211G→A) is the most common nonsynonymous change found in the East Asian population. The allele frequencies in Japanese, Korean, and Chinese populations have been reported to be 13, 23, and 23%, respectively (Akaba et al., 1998). It has been suggested that the G71R variant contributes to the high incidence of neonatal hyperbilirubinemia (Akaba et al., 1999). A comparable allele frequency (11%) has been reported for G71R mutation in Taiwanese population, where P229Q (686C→A) is found at a frequency of 2.8% (Huang et al., 2000). This allele frequency is comparable to that of Japanese patients with Crigler-Najjar syndrome type II (Yamamoto et al., 1998b). In the cDNA-transfected cells, the bilirubin glucuronidation activities of G71R and Y486D, normalized for the expression levels, have been shown to fall to 32 and 7.6% of that of WT, respectively (Yamamoto et al., 1998a). These values are comparable with the decrease in the SN-38 glucuronidation activity (normalized V_max) of G71R and Y486D found in this study (56 and 26% of WT, respectively; Table 1). However, there is a substantial large difference between the two substrates for the activities of P229Q. Koizumi et al. (1995) has reported that the bilirubin glucuronidation activity of P229Q decrease to 14% of that of WT (compared as a net activity), while, in our study, the V_max value of P229Q for the SN-38 glucuronidation activity was almost the same as that of WT. Thus, these mutations affect the glucuronidation activities in a substrate-specific manner, indicating that it is difficult to precisely predict the decreased SN-38 glucuronidation activities of UGT1A1 mutants from the reduction in the corresponding bilirubin glucuronidation activities. The normalized SN-38 glucuronidation efficiency ratio (normalized V_max/K_m in Table 1) decreased to 0.66 (47% of WT), 0.73 (52%), and 0.07 (5%) μmol/min/mg of protein for G71R, P229Q, and Y486D, respectively. Furthermore, the decreased expression of G71R and

**Discussion**

Although CPT-11 is a promising antitumor agent, severe, occasionally fatal, diarrhea, and/or leukopenia occur sporadically as the dose-limiting side effects of CPT-11. It has been suggested that the UGT1A1-mediated detoxification, or glucuronidation of SN-38 to
Y486D proteins were reproducibly observed without a significant reduction in their mRNA levels (Figs. 2 and 3), suggesting that the G71R and Y486D proteins are less stable or more rapidly degraded than the WT protein. If the decrease in the UGT1A1 protein expression level is a characteristic property of G71R and Y486D, it might be practical to use the net \( V_{\text{max}}/K_m \) values (19 and 2% of WT for G71R and Y486D, respectively) for estimating the influence of these mutations on the SN-38 glucuronidation activities. In this sense, both G71R and Y486D are significantly less active than WT and P229Q.

The clinical implications of the present study should be carefully interpreted. Since it has been established that TA polymorphism in the UGT1A1 promoter, which results in 30 to 80% reduction in expression, influences the incidence of severe toxicity by CPT-11 (Ando et al., 2000; Iyer et al., 2002), Y486D would certainly affect the pharmacokinetics/pharmacodynamics of SN-38 in vivo.

It is possible that G71R and P229Q mutations also affect the SN-38 glucuronidation in vivo by themselves or in combination with the extra TA insertion in the promoter. In accordance with this consideration, Ando et al. (2000) have reported in a case-control study that four of five patients (80%) who had the variant sequences both in the promoter (TA polymorphism) and in exon 1 (i.e., G71R or P229Q) suffered from life-threatening toxicity by CPT-11 infusion. Thus G71R and P229Q might considerably increase the susceptibility to CPT-11 when they coexist with other defective variant alleles.

Intriguingly another polymorphism has been found in the UGT1A1 promoter region. Sugatani et al. (2001) identified the 290-base pairs phenobarbital-responsive enhancer module (gtP-BREM) in the UGT1A1 gene promoter region (−3483/−3194), which was activated by the nuclear orphan receptor, human constitutive androstane receptor (hCAR). More recently, they have found a polymorphism (−3263T>G) in the gtP-BREM, which results in a reduction in gene expression to 60% of the wild-type gtP-BREM, as assessed by the luciferase-reporter assay (Sugatani et al., 2002). Therefore, the simultaneous detection of these changes in the UGT1A1 gene promoter and the coding region should be carried out to predict the CPT-11 toxicity.

In conclusion, among the three UGT1A1 variants investigated in this study, the SN-38 glucuronidation activity of Y486D is almost completely abolished, while the reduction in activity for G71R and P229Q is fractional. The decreased SN-38 glucuronidation efficiency ratio of G71R and P229Q could be critical in combination with other polymorphisms in the UGT1A1 gene. In the recent study of Gagné et al. (2002), the consistent decrease in the SN-38 glucuronidation activity was reported for G71R and Y486D variants. However, there is a discrepancy for the effect of P229Q: a residual activity of P229Q was 9% of WT in their report, while the normalized \( V_{\text{max}} \) value was approximately 80% of WT in the present study. At present, we have no explanation for this discrepancy, except for pointing to differences in cell types and/or experimental conditions. Further study will be required to clarify the influence of P229Q on the glucuronidation activity of SN-38.

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References


GLUCURONIDATION OF SN-38 BY WILD-TYPE AND VARIANT UGT1A1s