1521-009X/46/10/1446–1452$35.00
Drug Metabolism and Disposition

https://doi.org/10.1124/dmd.118.082495
Drug Metab Dispos 46:1446–1452, October 2018

Oligomerization and Catalytic Parameters of Human UDP-Glucuronosyltransferase 1A10: Expression and Characterization of the Recombinant Protein[S]

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Received May 17, 2018; accepted August 6, 2018

ABSTRACT

UDP-glucuronosyltransferase (UGT), as an integral membrane protein localized in the endoplasmic reticulum, has the ability to detoxify potentially hazardous xenobiotic substances. Most UGTs are expressed in liver, but UGT1A10 has proven to be an extrahepatic enzyme considerably expressed throughout the gastrointestinal tract. Earlier studies indicated that different UGT isoforms could exist in higher-order homo-oligomers or at least dimers within the membrane, but the formation of intermolecular disulfide bridges between UGT molecules was not often observed. In this study, we expressed recombinant human UGT1A10 in human embryonic kidney (HEK)293 and Chinese hamster ovary (CHO) cells to examine its oligomeric states and characterize its enzymatic activities against two therapeutically interesting substrates, morphine and entacapone, including determination of the catalytic rate constant (k_{cat}) values for the first time. It was observed that a majority of the UGT1A10 protein expressed in HEK293 cells existed in covalently crosslinked higher-order oligomers via formation of intermolecular disulfide bonds, whereas formation of the intermolecular disulfide bonds was not observed in the UGT1A10 protein expressed in CHO cells. Owing to the formation of the covalently crosslinked higher-order oligomers, the UGT1A10 protein expressed in HEK293 cells had much lower catalytic activity (particularly the catalytic rate constant k_{cat}) against both morphine and entacapone, compared with the UGT1A10 protein form expressed in CHO cells against the same substrates.

Introduction

Uridine 5′-diphospho-glucuronosyltransferases (UDP-glucuronosyltransferases, UGTs) are membrane-bound proteins localized in the endoplasmic reticulum (ER). These proteins catalyze the glucuronic acid transfer from UDP-glucuronic acids (UDP-GA) to small molecules (substrates). Human UGTs are divided into two gene families, UGT1 and UGT2, on the basis of evolutionary divergence (Meech and Mackenzie, 1997), and play a crucial role in detoxification and excretion of potentially hazardous xenobiotics as well as endogenous substances. However, it has been difficult to study the catalytic activity of an individual UGT in native tissues because many different UGTs are expressed in the same tissues, like liver, and their substrate specificities have frequently proven to be overlapping. Therefore, it has become common in the field to use recombinant enzymes exogenously expressed in different cell lines when testing whether a specific substrate of interest is converted by a UGT isomer into a glucuronide form and when comparing the catalytic efficiencies of UGT isoforms against a specific substrate. Added to this, the absence of a suitable method to purify UGTs as sufficiently active and mono-dispersed proteins has limited studies to the understanding of the structure-function relationships of these proteins.

Morphine remains the most valuable opioid analgesic for the management of moderate to severe pain (Wiffen et al., 2013). Morphine undergoes a considerable first-pass metabolism by UGTs in animals and humans after oral administration. The inactive morphine-3-glucuronide (M3G) and analgesically potent morphine-6-glucuronide (M6G) are the major metabolites of morphine in the body and they are mainly excreted by the urinary system (Déchelotte et al., 1993; Milne et al., 1996; Christrup, 1997). Although human liver still appears to be a prime organ responsible for the formation of morphine glucuronides (Milne et al., 1996), the respective contribution of the gastrointestinal tract and liver to the first-pass extraction of orally administrated morphine remained unclear (Déchelotte et al., 1993). In 2003, a systemic study of the recombinant human UGT isoforms related to morphine glucuronidation showed that UGT1A10, an extrahepatic enzyme restrictively expressed in the digestive tract, catalyzes the conversion of morphine to M3G, not M6G, at a relatively higher rate, compared with the other UGT1A isoforms tested (UGT1A1, 1A3, 1A6, 1A8, and 1A9) (Stone et al., 2003). However, the report only compared the measured V_{max} and K_M values of UGT isomers for the M3G formation, and there was no determination of the actual catalytic rate constant (k_{cat}) values, which limited the interpretation of the kinetic data with respect to the actual catalytic activities of individual UGTs.

In the present study, we first analyzed the expression of recombinant human UGT1A10 protein in 293 human embryonic kidney (HEK293)

This work was supported in part by the National Institutes of Health National Institute on Drug Abuse Translational Avant-Garde Award (UH2/UH3 DA041115) and Grants R01 DA035552, R01 DA032910, R01 DA013930, and R01 DA025100, and the National Science Foundation Grant CHE-1111761.
https://doi.org/10.1124/dmd.118.082495.
[S]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: CHO, Chinese hamster ovary; DTT, dithiothreitol; HEK293, 293 human embryonic kidney; HPLC, high-performance liquid chromatography; M3G, morphine-3-glucuronide; UDP-GA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

and Chinese hamster ovary (CHO) cells by Western blotting. We then kinetically characterized its glucuronidating activities with two different substrates, morphine and entacapone, the latter an inhibitor of catechol-*O*-methyltransferase (COMT) used in the treatment of Parkinson's disease. The kinetic characterization has allowed us to determine the k_{cat} values of UGT1A10 against morphine and entacapone for the first time. We also demonstrated that recombinant human UGT1A10 protein expressed in HEK293 cells forms oligomerized complexes that are covalently crosslinked by disulfide bonds but when expressed in CHO cells barely forms crosslinked disulfide bonds. In addition, the complete catalytic parameters obtained for membrane-bound UGT1A10 against morphine and entacapone reveal that the catalytic activities of recombinant UGT1A10 proteins are remarkably different, depending on the type of cell line used to express the protein.

Materials and Methods

Chemicals and Materials. Phusion DNA polymerases, restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All oligonucleotides were purchased from Eurofins MWG Operon (Louisville, KY). FreeStyle Chinese hamster ovary-S cells (denoted as CHO for convenience), FreeStyle 293 human embryonic kidney cells (denoted as HEK293 for convenience), HEK293FT, FreeStyle CHO Expression Medium, FreeStyle 293 Expression Medium, hypoxanthine/thymidine (HT) supplement, L-glutamine, 4%–12% Tris-glycine Mini Protein Gel, and SimpleBlue SafeStain were purchased from Life Technologies (Carlsbad, CA). Morphine was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program. Morphine-3-glucuronide, entacapone, entacapone 3-*O*-glucuronide, UDP-GA, Triton X-100, saccharolactone, β-glucuronidase, phospholipids (phosphatidylcholine type X-E), and the solvents used in high-performance liquid chromatography (HPLC) were purchased from MilliporeSigma (St. Louis, MO). HisPur Cobalt Resin was obtained from Thermo Fisher Scientific (Waltham, MA). Centrifugal filter units were ordered from Millipore (Burlington, MA).

Generation of the Stable Cell Line by Lentivirus Infection. Cell lines stably overexpressing human UGT1A10 were generated using a lentivirus-based method described in our previous report (Xue et al., 2013). Briefly, the human UGT1A10-6xHis gene was first synthesized by GenScript Corporation (Piscataway, NJ) on the basis of the published sequence in GenBank (NM_019075.2) and inserted in the pCSC-SP-PW vector, lentivirus plasmid. To package the lentivirus particles carrying UGT1A10-6xHis gene, lentivirus was produced by cotransfecting pCSC-human UGT1A10-6xHis plasmid with the two packaging vectors (pMDLg/pRRE and pRSV-Rev) and one envelope plasmid (pCMV-VSV-G) into FreeStyle HEK293 cells transformed with the SV40 large T antigen (denoted as HEK293T for convenience) by lipofection. CHO and HEK293 cells were then transduced with the packaged lentivirus particles. The infected cells were recovered from the infection for 2 days or more and transferred to a shake flask for scaled-up culture. The stable cell pools obtained were kept frozen before use.

Microsomal Preparation. Cells were washed with Tris-buffered saline (25 mM Tris base, pH 7.4, 138 mM NaCl, and 2.7 mM KCl) followed by a centrifugation at 2000*g* for 5 minutes at 4°C. The cell homogenates were prepared by resuspending the cell pellets in 25 mM Tris-Cl, pH 7.4, and subjecting them to sonication. To remove cell debris or unbroken cells, the total cell homogenates were exposed to centrifugation at 10,000*g* for 20 minutes. Microsomes were prepared by ultracentrifugation of the supernatant at 100,000*g* for 1.5 hours and resuspending the resulting microsomal fraction in 25 mM Tris-Cl, pH 7.4. Microsomes (10 mg protein/ml) were stored at −70°C before use and their concentrations were determined using the Bradford assay from Thermo Fisher Scientific.

Purification of UGT1A10. The purification of recombinant human UGT1A10-6xHis protein followed a method described by Kurkela et al. (2003), except that a HisPur Cobalt Resin from Thermo Fisher Scientific was used rather than a nickel-charged His Hi-Trap column from GE Healthcare Life Sciences (Pittsburgh, PA). Briefly, microsomes were suspended in an extraction buffer (25 mM Tris, pH 7.4, 500 mM NaCl, and 1% Triton X-100) at 2 mg/ml of concentration followed by incubation for 10 minutes with shaking at 4°C. The suspension was centrifuged at 100,000*g* for 1 hour The resultant supernatant was loaded onto a HisPur Cobalt Resin (Thermo Fisher Scientific) which had been pre-equilibrated with a washing buffer (25 mM Tris, pH 7.4, 500 mM NaCl, 0.05% Triton X-100, and 50 mM imidazole). After extensive washing with the washing buffer, bound His-tagged proteins were eluted by a stepwise gradient elution with imidazole in the presence of 0.05% Triton X-100 and 150 mM NaCl. The eluents were analyzed by SDS-PAGE and Western blot for UGT1A. The concentration of purified UGT1A10-6xHis protein was determined using the Bradford assay (Thermo Fisher Scientific).

Enzyme Activity Assays. Enzymatic glucuronidation of morphine or entacapone was tested under the following assay conditions. All enzyme assays (100-μl final volume) contained 0.1 M phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 5 mM saccharolactone, 5 mM UDP-GA, and 100 μg of microsomal protein or 50 ng of purified UGT1A10-6xHis. Phospholipid (1 mg/ml) was added to the assay mixtures containing the purified UGT1A10-6xHis. The concentrations of aglycone substrate ranged from 0.1 to 20 mM (for morphine) or from 3 to 2000 μM (for entacapone). To initiate the reactions, UDP-GA (5 mM in incubation) was added to give a 100-μl final volume and then the reactions were incubated at 37°C with shaking. The reaction time was adjusted so that no more than 10% of substrate was depleted during reaction. Blank incubations were performed in the same manner but without UDP-GA. The reaction was terminated with 100 μl of glycine-HCl (pH 2) containing 1% (v/v) Triton X-100. The stopped reactions were centrifuged to pellet precipitated protein. The resulting supernatants were subjected to reverse-phase HPLC (RP-HPLC) on a 5-μm C18 110 Å column (250 × 4.6 mm, Gemini; Life Technologies/Thermo Fisher Scientific), and RP-HPLC was performed using the mobile phase consisting of 10% acetonitrile in 0.1% trifluoroacetic acid and the remaining substrate and resulting products were monitored by UV absorbance at 230 nm (morphine and its glucuronide) or 315 nm (entacapone and its glucuronide). The basis of quantification was a standard curve prepared using an authentic standard compound. All samples were prepared in duplicate or triplicate. GraphPad Prism 5.01 software (La Jolla, CA) was used to analyze the kinetic data.

Hydrolysis by β-Glucuronidase. The reaction mixture was centrifuged at 13,000*g* for 10 minutes and the resultant supernatant was transferred to a new 1.5-ml microcentrifuge tube. β-Glucuronidase was added to the supernatant at final concentration of 4 IU/ml. The sample was incubated at 37°C for 2 hours before the reaction was terminated by 100 μl glycine-HCl (pH 2) containing 1% (v/v) Triton X-100.

Western Blot Assay. The levels of membrane-bound UGT1A10 protein in microsomes prepared from human UGT1A10-6xHis–overexpressing cell lines were measured by Western blot analysis using mouse anti-human UGT1A IgG obtained from Santa Cruz Biotechnology (Dallas, TX) (1:3000 dilution as described in the manufacturer's instructions). Horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was used at 1:4000 as a secondary antibody and UGT1A10 protein was finally detected by chemiluminescence using the SuperSignal West Dura Extended Duration Substrate from (Pierce; Thermo Fisher Scientific). The levels of UGT1A10 were further quantified against a known amount of purified UGT1A10-6xHis protein by densitometric scanning of the blots using Quantity One software (Bio-Rad, Hercules, CA).

Results

Overexpression of Human UGT1A10 in CHO and HEK293 Cells. When expressed in CHO and HEK293 cells, recombinant human UGT1A10 protein was detected mostly at ~65 kDa (monomeric size) by immunoblotting with an anti-UGT1A antiserum. After longer exposure, UGT1A10 protein was also observed as unexpected bands at approximately ~130 kDa, and higher than ~130 kDa in both microsomes prepared from UGT1A10-6xHis–overexpressing stable CHO or HEK293 cells (three independent Western blotting tests showed the same bands) (Fig. 1A). The bands with molecular weight higher than ~65 kDa are neither endogenous UGT1A nor UGT2B isoforms, as they were not detected in the microsomal proteins of native CHO and HEK293 cells by immunoblotting with anti-UGT1A or UGT1B antiserum (data not shown), which indicates that the unexpectedly high molecular-weight bands were extremely stable

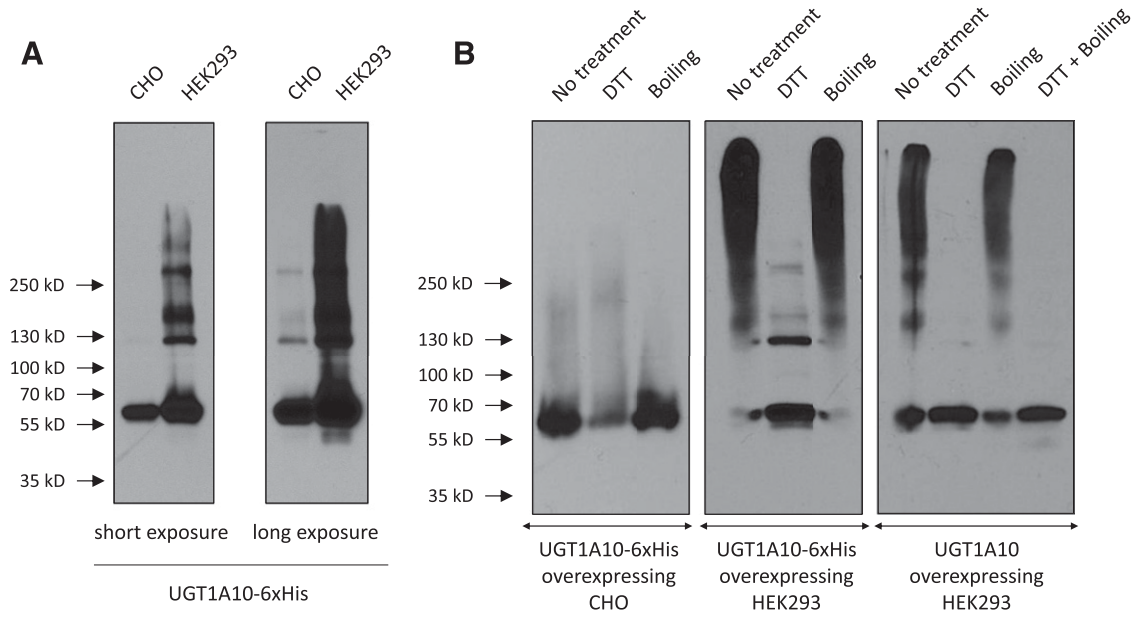

Fig. 1. Analysis of recombinant UGT1A10 expression. (A) Western blot analysis of UGT1A10 protein in microsomes prepared from UGT1A10-6xHis-overexpressing stable CHO and HEK293 cell lines. Microsomes were boiled at 95°C for 10 minutes in the presence of 100 mM DTT prior to electrophoresis. (B) SDS-PAGE of microsomes under different denaturating conditions, followed by immunoblot analysis. DTT, electrophoresis after reduction with 100 mM DTT; Boiling, electrophoresis after boiling; No treatment, no DTT and boiling before electrophoresis.

homo-oligomers of UGT1A10 or hetero-oligomeric complexes with another protein.

Previously, Matern et al. (1982) showed that a form of active rat UGT extracted from rat liver appears with an apparent molecular mass of 316 kDa, and the subunit molecular weight was determined as ~54 kDa, suggesting that the formation of oligomeric UGT complexes occurred naturally or inadvertently after purification. In line with this, recent reports also support (Ghosh et al., 2001; Operaña and Tukey, 2007; Finel and Kurkela, 2008) the existence of UGT proteins in the membrane tissue as higher-order oligomers (at least dimers). These observations led us to test whether the majority of recombinant human UGT1A10 enzyme in CHO and HEK293 cells are also highly organized within the membrane of ER and exist as homo-oligomeric complexes. For this purpose, UGT1A10 proteins in microsomes prepared from the stable cell lines indicated above were first exposed to different denaturating conditions then separated on SDS-PAGE followed by immunoblot detection with anti-UGT1A antibody as described in *Materials and Methods*. We found that the majority of HEK293-expressed UGT1A10 ($UGT1A10^{HEK293}$) enzyme molecules formed the higher-order oligomers that were completely resolved to the monomeric size of UGT1A10

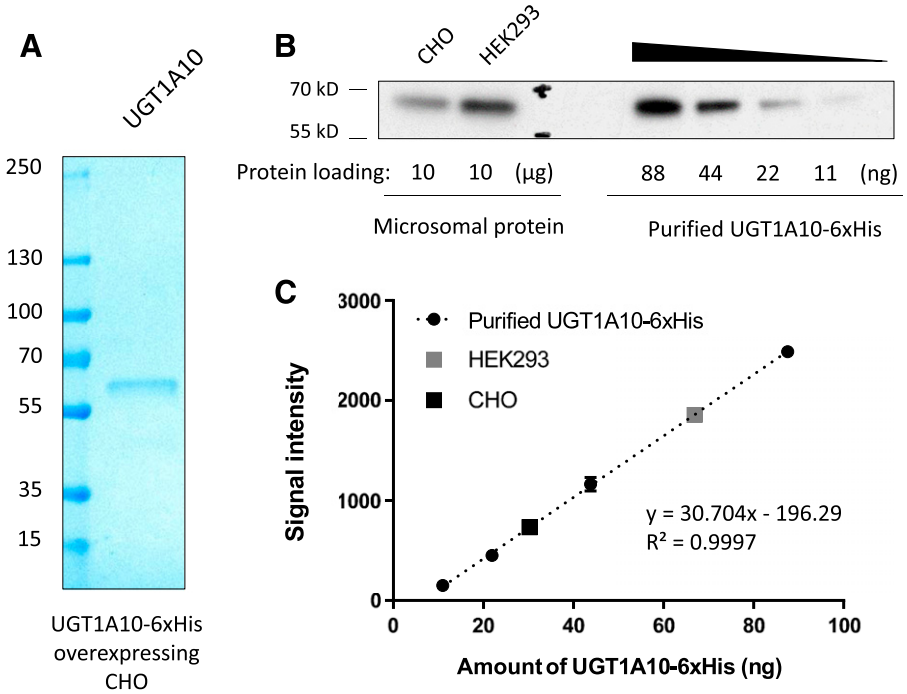

Fig. 2. Quantification of the levels of UGT1A10 in microsomes. (A) SDS-PAGE analysis of pure UGT1A10-6xHis extracted from microsome prepared from UGT1A10-6xHis-overexpressing stale CHO cells. The sizes of the molecular mass markers are indicated on the left in kilodaltons. (B) Chemiluminescent blot of dilution series of purified UGT1A10-6xHis and two unknown amounts of membrane-bound UGT1A10-6xHis. (C) The linear dynamic range of film-based detection for UGT1A10-6xHis. The graph shows a quantitative level of UGT1A10-6xHis protein for the corresponding chemiluminescence signal intensity. Each dot represents the average of duplicates and its values are expressed as the mean ± S.D.

upon SDS-PAGE after treatment with dithiothreitol (DTT) and the minority migrate as dimers after reduction. However, the covalently crosslinked higher-order oligomers were not observed in the CHO-expressed UGT1A10 ($UGT1A10^{CHO}$). Boiling the samples neither formed aggregates of UGT1A10 proteins nor made changes in the results (Fig. 1B). To examine whether the oligomeric UGT1A10 complexes seen in the stable HEK293 cells are homomeric or heteromeric, anti-UGT1A immunoprecipitation was performed. However, whereas $UGT1A10^{CHO}$ enzyme was efficiently precipitated, $UGT1A10^{HEK293}$ enzyme was not in the same experimental condition (Supplemental Fig. 1).

Quantification of the Levels of Recombinant UGT1A10 in Microsomes Prepared from Stable Cells. Considering that disulfide bridges contribute considerably to the interaction of UGT1A10 expressed in HEK293, but not in CHO cells, we questioned whether the UGT1A10 enzyme activity may be altered by the formation of intermolecular crosslinks via disulfide bonds between UGT1A10 molecules. To address the question, we first determined the concentrations of UGT1A10 expressed using the two stable cell lines, and then their kinetic parameter values (k_{cat} and K_M) were evaluated and compared in subsequent kinetic assays. As shown in Fig. 2A, we purified UGT1A10 from microsome prepared from the UGT1A10-6xHis-overexpressing stable CHO cells and determined its concentration as noted in *Materials and Methods*. Differing amounts of purified UGT1A10 were then loaded as indicated to obtain a single blot with densitometric readings on the linear part of the curve for membrane-bound UGT1A10 in microsomes (Fig 2, B and C). The concentrations of membrane-bound UGT1A10 in microsomes prepared from the stable HEK293 and CHO cells were determined to be 6.69 and 3.02 ng/μg of microsomal proteins, respectively.

Kinetics of Morphine Glucuronidation by Recombinant Human UGT1A10. To test whether HEK293- and CHO-expressed UGT1A10 protein forms have similar catalytic activity against morphine, we investigated the kinetics of the formation of morphine-3-glucuronide catalyzed by UGT1A10. As shown in a representative of HPLC chromatograms depicting the peak for M3G (Fig. 3A), the glucuronidation activity was observed for both membrane-bound $UGT1A10^{CHO}$ or $UGT1A10^{HEK293}$ enzymes against morphine (top and middle), and the peak (M3G) with a retention time of 5.4 minutes disappeared after β-glucuronidase was added to the reaction (bottom). The generated kinetic data are depicted in Fig. 3B, and the kinetic parameters obtained are summarized in Table 1. As shown in Table 1, compared with membrane-bound $UGT1A10^{HEK293}$, membrane-bound $UGT1A10^{CHO}$ had a higher k_{cat} value (8.26 $minutes^{-1}$ compared with 0.90 $minutes^{-1}$) and a slightly lower K_M value (7.3 mM compared with 10.4 mM). These data indicate that the major difference between membrane-bound $UGT1A10^{CHO}$ and $UGT1A10^{HEK293}$ in the catalytic efficiency against morphine ($k_{cat}/K_M = 1.13 \times 10^3\ min^{-1} \cdot M^{-1}$ for $UGT1A10^{CHO}$ vs. $k_{cat}/K_M = 86.51 min^{-1} \cdot M^{-1}$ for $UGT1A10^{HEK293}$) is largely attributable to their turnover numbers (k_{cat}) for the M3G formation. However, glucuronidation of morphine by the purified UGT1A10 was not observed under the experimental conditions generating the kinetic data depicted in Fig. 3B.

Kinetics of Entacapone Glucuronidation by Recombinant Human UGT1A10. Entacapone is an inhibitor of catechol-*O*-methyltransferase used in the treatment of Parkinson's disease. Entacapone is known to be metabolized into entacapone 3-*O*-glucuronide by different UGTs. As shown in Fig. 4A, the peak for entacapone 3-*O*-glucuronide (retention time = 9.9 minutes) appeared in the presence of membrane-bound $UGT1A10^{CHO}$ or $UGT1A10^{HEK293}$ (top and middle) enzyme, and the peak was no longer found if the reaction was further incubated with β-glucuronidase (bottom), indicating that

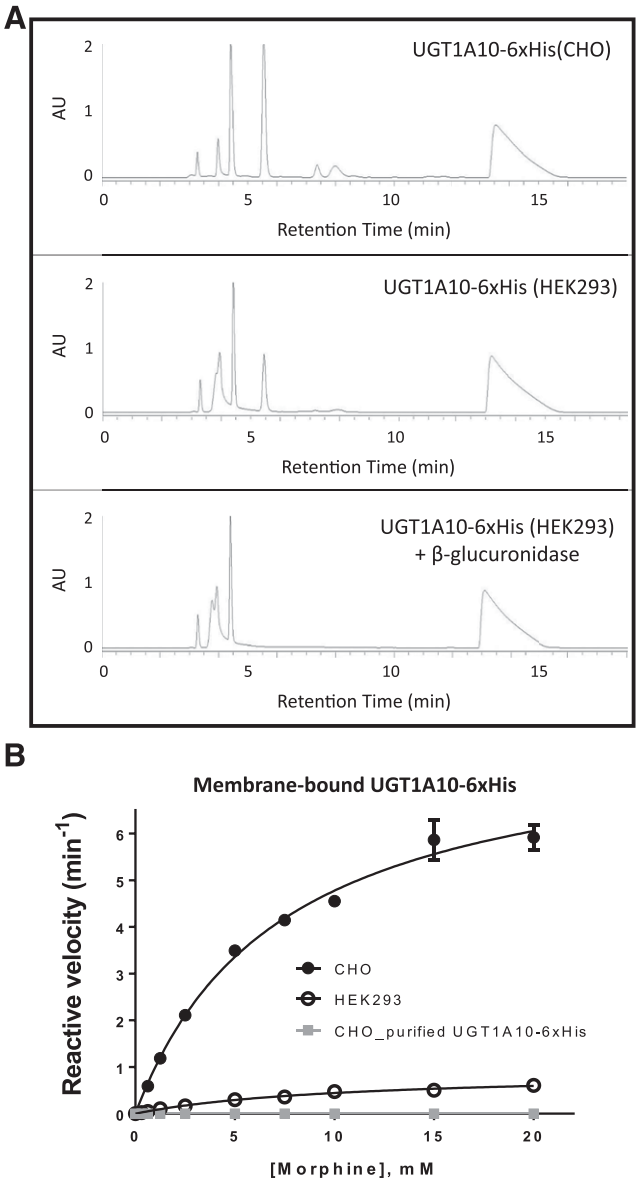

Fig. 3. Kinetic analysis of the formation of M3G by UGT1A10. (A) HPLC analysis of M3G formation using microsomes prepared from UGT1A10-6xHis-overexpressing stable cell lines. Top, membrane-bound UGT1A10-6xHis expressed in CHO cells; middle, membrane-bound UGT1A10-6xHis expressed in HEK293 cells; bottom, membrane-bound UGT1A10-6xHis expressed in HEK293 cells with treatment with β-glucuronidase. (B) M3G formation by membrane-bound UGT1A10-6xHis enzymes in CHO cells (●) or in HEK293 cells (○), or by purified UGT1A10-6xHis protein (■) were determined at substrate concentrations of 0.1–20 mM. Kinetic parameters (k_{cat} and K_M) were determined by fitting the measured reaction rate data to the Michaelis-Menten kinetic equation using the Prism5.01 software. Each dot is the representative of the average of triplicates and its values are expressed as the mean ± S.D.

entacapone was recognized by both the membrane-bound UGT1A10 enzyme forms as a substrate. To assess whether the observed differences in the catalytic activity against morphine between membrane-bound $UGT1A10^{CHO}$ and $UGT1A10^{HEK293}$ are also manifested against a different substrate, an additional kinetic assay was performed against entacapone. The kinetic data obtained are depicted in Fig. 4B, and the kinetic parameters determined are summarized in Table 2. As shown in Table 2, the expected similar differences in glucuronidation kinetics for entacapone were also observed between membrane-bound $UGT1A10^{CHO}$ and $UGT1A10^{HEK293}$. A substantially higher catalytic

TABLE 1
Kinetic parameters of human UGT1A10-6xHis against morphine

UGT1A10-6xHis	K_M	k_{cat}	k_{cat}/K_M	RCE[a]	R^2
	mM	*min*$^{-1}$	*min*$^{-1}$ · *M*$^{-1}$		
Membrane-bound_HEK293	10.4 ± 0.96	0.90 ± 0.04	86.5	1	0.994
Membrane-bound_CHO	7.30 ± 0.81	8.26 ± 0.38	1.13×10^3	13.0	0.988

[a]Relative catalytic efficiency (k_{cat}/K_M).

efficiency against entacapone was observed for membrane-bound UGT1A10^{CHO} compared with UGT1A10^{HEK293} (5.53×10^5 min^{-1} · M^{-1} compared with 7.71×10^4 min^{-1} · M^{-1}) and this difference is mainly owing to a substantially higher k_{cat} value (25.3 minutes^{-1} compared with 4.39 minutes^{-1}) and a slightly lower K_M value (45.7 μM compared with 56.9 μM) of membrane-bound UGT1A10^{CHO} compared with membrane-bound UGT1A10^{HEK293}. Added to this, although the purified UGT1A10 showed a detectable enzymatic activity toward entacapone, the experimental K_M and k_{cat} values of membrane UGT1A10^{CHO} were indeed increased by a factor of about 15 (45.7 μM compared with 703 μM) and decreased by a factor of about 320 (25.3 minutes^{-1} compared with 0.079 minutes^{-1}), respectively, during the purification process. Moreover, it was observed that the rate constant of entacapone glucuronidation by the purified UGT1A10 was only slightly increased by the addition of phospholipid sonicated (Fig. 4C).

Discussion

An extrahepatic human UGT1A10 was expressed as an active enzyme using lentivirus-infected HEK293 and CHO cells. Our recombinant UGT1A10 has a C-terminal hexa-histidine tag, which allows for efficient single-step chromatographic purification using immobilized-metal affinity chromatography. Indeed, human recombinant UGTs containing an His tag at the C-terminus have been widely used for the enzymatic characterization, structure determination, and substrate screening studies of the UGTs (Kurkela et al., 2003; Little et al., 2004; Radominska-Pandya et al., 2005; Kato et al., 2013; He et al., 2018). Zhang et al. (2012) demonstrated only a mild increase in the K_M values in UGT1A9 and 2B7 containing the C-terminal His-tag but no differences in parameters such as those used in the kinetic model. Consistent with their findings, we found that CHO-expressed recombinant UGT1A10 proteins displayed similar K_M values for morphine [K_M = 7.30 ± 0.81 mM for UGT1A10-6xHis and K_M = 6.60 ± 0.33 mM for UGT1A10 (data not shown)], regardless of the addition of an His tag to the C-terminal end, which suggests that UGT1A10-6xHis is a good model for the functional studies.

Previously, Kurkela et al. (2003) reported a good method to purify human UGT1A9 as an active form using Triton X-100. In our study, we demonstrated that their method also works for purification of active UGT1A10, which shares about 93.2% identical protein sequence with UGT1A9. However, the purification process caused an irreversible and considerable decrease in UGT1A10 enzymatic activity, which cannot be compensated for merely by phospholipid addition. Actually, this observation is consistent with the findings of Kurkela et al. (2003). Compared with membrane-bound UGT1A10^{CHO}, the K_M and k_{cat} values for entacapone were reduced ~15 and ~320 times, respectively, during the purification process.

The oligomeric states of the UGTs have been studied for more than two decades. One of the main reasons for the attempts was to determine whether the enzymatic activities of the UGTs are affected by their oligomeric states. The two previous studies of pure UGT isoforms extracted from the native tissues showed that UGTs could be present as tetramers or even higher-order oligomers (Tukey and Tephly, 1981; Matern et al., 1982). By nearest-neighbor crosslinking and yeast two-hybrid analysis, Ghosh et al. (2001) also revealed that recombinant human UGT1A1 enzymes within microsomal membrane form homo-oligomers. Added to this, a study using fluorescence resonance energy transfer as a tool to demonstrate oligomerization of UGT1A7 proteins (Operaña and Tukey, 2007) reported that ~90% of UGT1A7 proteins existed as homo-oligomeric complexes in live cells. In this study, we

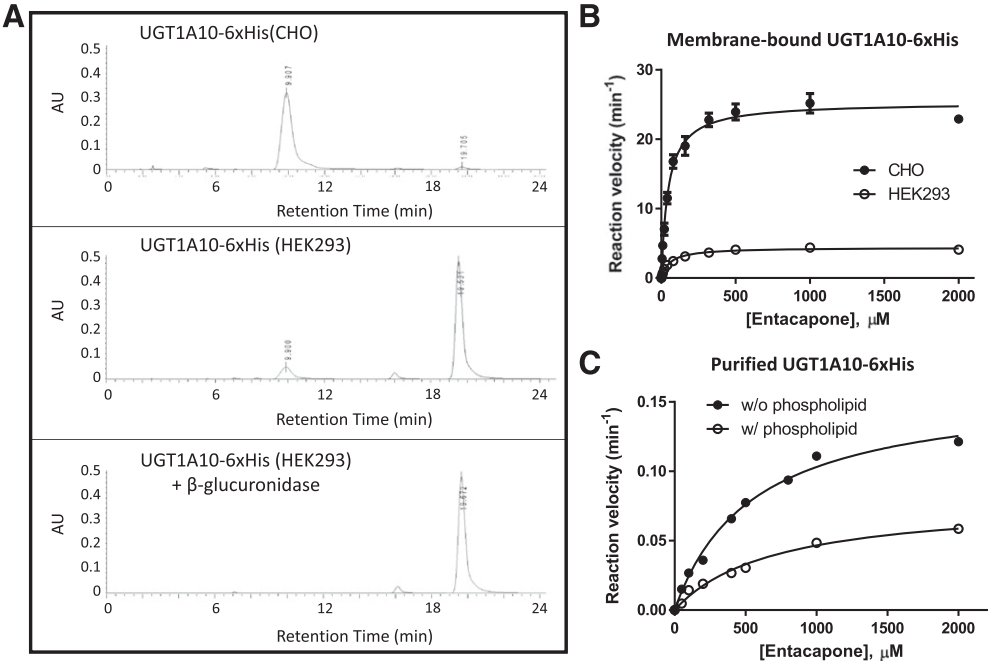

Fig. 4. Kinetic analysis of entacapone 3-*O*-glucuronide formation by UGT1A10. (A) HPLC analysis of the formation of entacapone 3-*O*-glucuronide using microsomes prepared from UGT1A10-6xHis-over-expressing stable cell lines. Top, membrane-bound UGT1A10-6xHis expressed in CHO cells; middle, membrane-bound UGT1A10-6xHis expressed in HEK293 cells; bottom, membrane-bound UGT1A10-6xHis expressed in HEK293 cells with treatment with β-glucuronidase. (B) Formation of entacapone 3-*O*-glucuronide by membrane-bound UGT1A10-6xHis protein in CHO cells (●), or in HEK293 cells (○) were determined at substrate concentrations of 3–2000 μM. (C) Formation of entacapone 3-*O*-glucuronide by purified UGT1A10-6xHis protein was determined at substrate concentrations of 3–2000 μM in the presence (●) or absence (○) of 1 mg/ml of phosphatidylcholine type X-E. Kinetic parameters (k_{cat} and K_M) were determined by fitting the generated reaction rate data to the Michaelis-Menten kinetic equation using the Prism5.01 software. Each dot refers to the average of duplicate or triplicates and its values are expressed as the mean ± S.D.

TABLE 2

Kinetic parameters of human UGT1A10-6xHis against entacapone

UGT1A10-6xHis	K_M	k_{cat}	k_{cat}/K_M	RCE[a]	R^2
	μM	min^{-1}	$min^{-1} \cdot M^{-1}$		
Membrane-bound_HEK293	56.9 ± 4.1	4.39 ± 0.07	7.71×10^4	1	0.987
Membrane-bound_CHO	45.7 ± 4.5	25.3 ± 0.6	5.53×10^5	7.17	0.992
Purified_CHO w/o phospholipid	703 ± 116	0.079 ± 0.005	1.12×10^2	1.45×10^{-3}	0.991
Purified_CHO w/ phospholipid	554 ± 72	0.16 ± 0.01	2.88×10^2	3.74×10^{-3}	0.995

[a]Relative catalytic efficiency (k_{cat}/K_M).

found that most recombinant human UGT1A10 enzymes expressed in HEK293 cells formed the covalently crosslinked higher-order oligomers via intermolecular disulfide bonds (Fig. 1B). To the best of our knowledge, this is not only the first demonstration of the oligomerized UGT1A10 complexes but also the first evidence of the presence of complicated disulfide bridges in the formation of higher-order UGT complexes bigger than a dimer. Considering that the covalently crosslinked homo-oligomers are not frequently observed in recombinant human UGT1A1, 1A4, and 1A6 enzymes expressed in HEK293 cells, as reported by Fujiwara et al. (2007), the observed multiple disulfide bonds formed in the oligomerized UGT1A10 complexes seem to be unique.

Interestingly, we also found that CHO-expressed UGT1A10 was barely crosslinked via disulfide bridges, unlike the HEK293-expressed UGT1A10 (Fig. 1). This finding led us to ask whether there is any difference in activity between these UGT1A10 enzyme forms. Our kinetic assays on the HEK- and CHO-expressed UGT1A10 enzyme forms for their catalytic activities against morphine and entacapone revealed that HEK293-expressed UGT1A10 had a similar K_M value, but a substantially decreased k_{cat} value, compared with the CHO-expressed UGT1A10. These findings suggest that the intermolecular disulfide bonds in the HEK293-expressed UGT1A10 protein would substantially decrease the catalytic activities of the enzyme against both of the substrates (Tables 1 and 2). However, since were used the UGT1A10 enzyme forms in the microsomal fractions, not the purified enzyme forms, for our enzyme activity assays, we still cannot rule out completely alternative possibilities, such as the presence of another key determinant of UGT1A10 enzyme activity. Indeed, we also tested whether an enzymatic activity of HEK293-expressed UGT1A10 can increase by disrupting the S-S bonds (Supplemental Fig. 2). It was observed that both membrane-bound $UGT1A10^{HEK293}$ and $UGT1A10^{CHO}$ became inactive after incubation with 100 mM DTT for 1 hour, which strongly suggests that the intramolecular disulfide bonds of UGT1A10 enzyme are crucial for its activity or stability. Despite of the experimental limitation, our observations clearly indicate that the enzymatic activity and post-translational modification of UGT1A10 can be significantly affected by the cell line used to express the protein. In addition, according to the modeled van der Waals surface of the UGT1A10 protein structure (Supplemental Fig. 3), UGT1A10 has three cysteine residues (C72, C183, and C277) on the protein surface. C183 of one UGT1A10 molecule could form a disulfide bond with C72 or C277 of another UGT1A10 molecule, i.e., forming intermolecular disulfide bonds. Further, there are multiple asparagine residues (that are potential glycosylation sites) near C72 and C277 on the protein surface. Different glycan structures formed in different types of cells could have differential effects on formation of the intermolecular disulfide crosslinks, which helps us to understand the observed remarkable oligomerization difference between the two cell lines.

Considering that a nonhepatic UGT1A10 enzyme is substantially expressed in human small intestine and colon with UGT1A1, 2B7, 2B15, and 2B17 (Nakamura et al., 2008; Ohno and Nakajin, 2009; Sato et al., 2014; Oda et al., 2017), there still remains notable interest in the potential contributions of these enzymes to the first-pass metabolism of morphine in the gastrointestinal tract after oral uptake. This is the first report of the complete kinetic parameters (k_{cat} and K_M) of UGT1A10 against morphine and entacapone. Extending the lines of this study using the UGTs mentioned above will help to enhance our understanding of their significant contributions to the first-pass extraction of orally administered drugs, including morphine.

Acknowledgments

Chemicals including morphine were kindly provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program. We thank Dr. Zhenyu Jin for laboratory assistance and Drs. Yaxia Yuan and Kuo-Hao Lee for their help on molecular modeling.

Authorship Contributions

Participated in research design: Zheng, Zhan.
Conducted experiments: Kim.
Contributed new reagents or analytic tools: Kim.
Performed data analysis: Kim.
Wrote or contributed to the writing of the manuscript: Kim, Zheng, Zhan.

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Supplemental Data

Oligomerization and catalytic parameters of human UDP-glucuronosyltransferase 1A10: Expression and characterization of the recombinant protein

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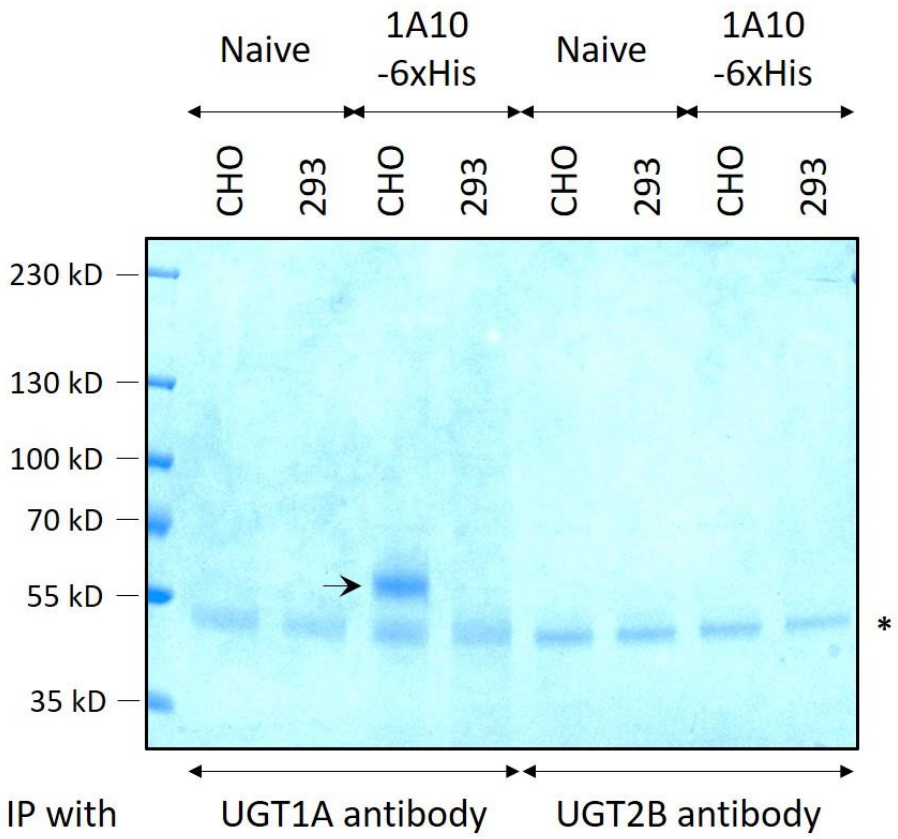
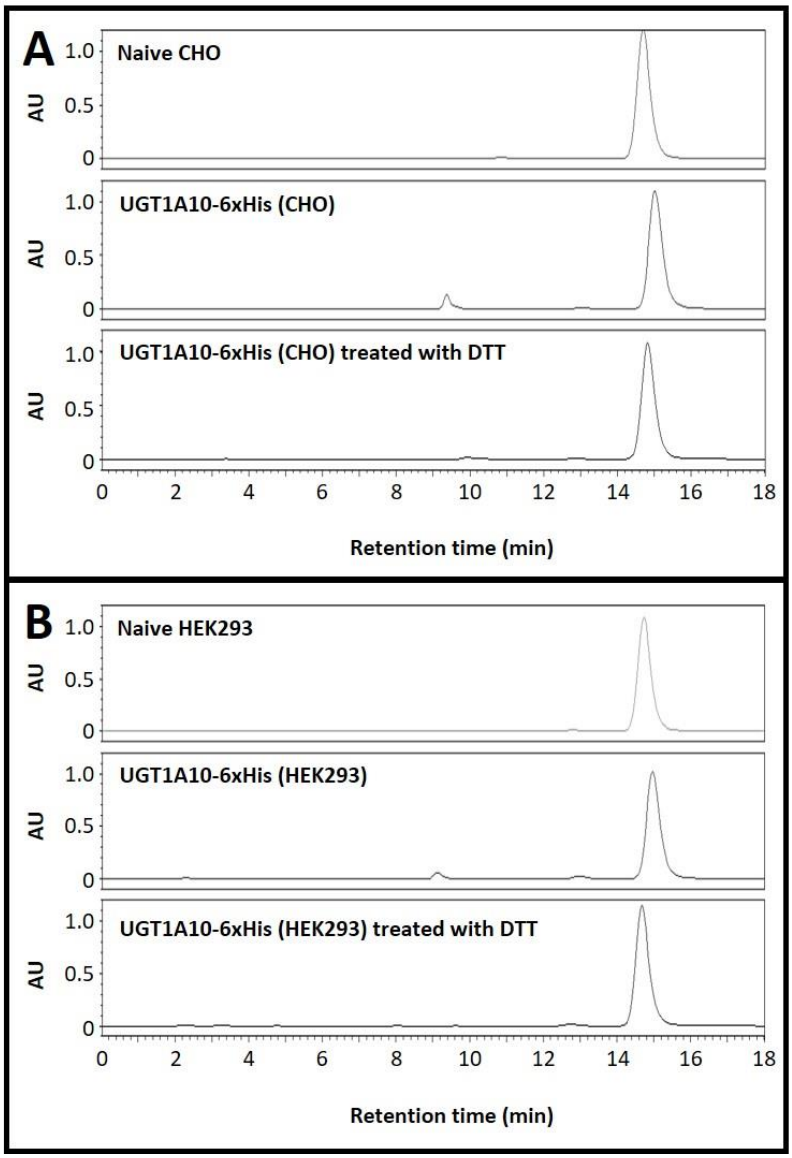
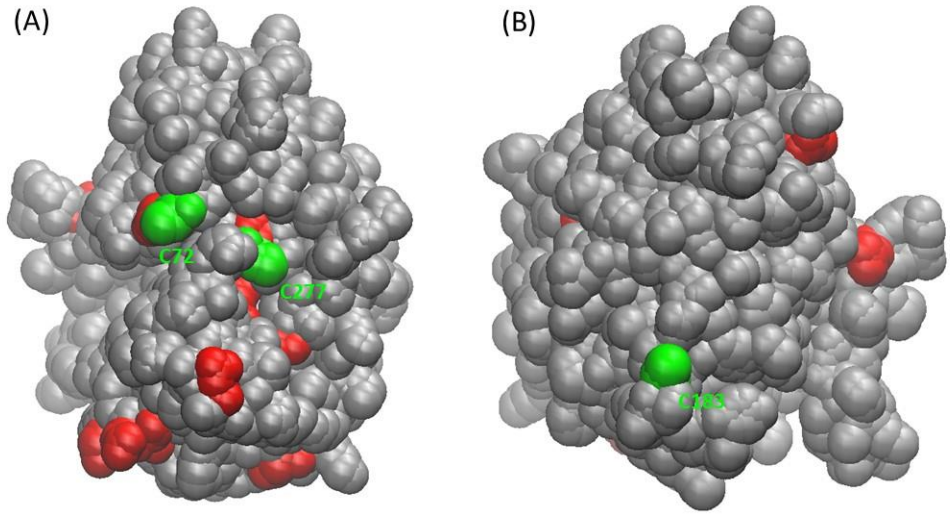


Supplemental Figure 1. Immunoprecipitation assay for UGT1A10-6xHis. Membrane-bound UGT1A10-6xHis protein was first extracted with 1.5% Triton X-100, followed by anti-UGT1A immunoprecipitation. Precipitated UGT1A10-6xHis was revealed by coomassie blue staining (see arrow). Asterisk marks the heavy chain of the immunoglobulin and this was used as an internal loading control.



Supplemental Figure 2. HPLC analysis of the formation of entacapone 3-*O*-glucuronide using microsomes prepared from UGT1A10-6xHis-overexpressing stable cell lines. Retention time was found to be about 9.4 min and 15 min for entacapone 3-*O*-glucuronide and entacapone. (A) Top, microsomal proteins prepared from naïve CHO cells; middle, membrane-bound UGT1A10-6xHis expressed in CHO cells; bottom, membrane-bound UGT1A10-6xHis expressed in CHO cells was incubated with 100 mM DTT for 1 h prior to glucuronidation activity assay. (B) Top, microsomal proteins prepared from naïve HEK293 cells; middle, membrane-bound UGT1A10-6xHis expressed in HEK293 cells; bottom, membrane-bound UGT1A10-6xHis expressed in HEK293 cells was incubated with 100 mM DTT for 1 h prior to glucuronidation activity assay.



Supplemental Figure 3. The modeled van der Waals surface of the UGT1A10 protein structure, showing three solvent-accessible cysteine residues (C72, C183, and C277 in green color). Panels A and B show two sides of the protein, with C72 and C277 on one side and C183 on the opposite side. Indicated in red color are solvent-accessible asparagine residues (potential glycosylation sites). C72 and C277 are close to the entrance of the enzyme active-site pocket. C72 or C277 of a UGT1A10 molecule may form a disulfide bond with C183 or C72 or C277 of another UGT1A10 molecule for crosslinking *via* intermolecular disulfide bonds. The crosslinking disulfide bonds involving C72 or C277 are expected to block the entrance of the enzyme active-site pocket and, thus, decrease the catalytic activity of the enzyme.