Heterodimerization of human UDP-glucuronosyltransferase (UGT)
1A9 and UGT2B7 alters their glucuronidation activities

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Heterodimerization of UGT1A9 and UGT2B7

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Abbreviations

AZT, zidovudine; AZTG, zidovudine O-glucuronide; CFP, cyan fluorescent protein; Co-IP, co-immunoprecipitation; CT, cytosolic tail; CTD, C-terminal domain; CV, column volumes; DM, di-lysine motif; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; HA, haemagglutinin; IS, internal standard; NTD, N-terminal domain; PG, propofol O-glucuronide; PMT, photomultiplier tube; PTM, post-translational modification; SD, standard deviation; Sf9, Spodoptera frugiperda; SNPs, single nucleotide polymorphisms; SP, signal peptide; TM, transmembrane; UDPGA, UDP-glucuronic acid; UGTs, UDP-glucuronosyltransferases; WT, wild-type; YFP, yellow fluorescent protein.
Abstract

Human UDP-glucuronosyltransferases (UGTs) play a pivotal role as prominent phase II metabolic enzymes, mediating the glucuronidation of both endobiotics and xenobiotics. Dimerization greatly modulates the enzymatic activities of UGTs. In this study, we examined the influence of three mutations (H35A, H268Y, and N68A/N315A) and four truncations (signal peptide, single transmembrane helix, cytosolic tail, and di-lysine motif) in UGT2B7 on its heterodimerization with wild-type UGT1A9, using a Bac-to-Bac expression system. We employed quantitative fluorescence resonance energy transfer (FRET) techniques and co-immunoprecipitation (Co-IP) assays to evaluate the formation of heterodimers between UGT1A9 and UGT2B7 allozymes. Furthermore, we evaluated the glucuronidation activities of the heterodimers using zidovudine and propofol as substrates for UGT2B7 and UGT1A9, respectively. Our findings revealed that the histidine residue at codon 35 was involved in the dimeric interaction, as evidenced by the FRET efficiencies and catalytic activities. Interestingly, the signal peptide and single transmembrane helix domain of UGT2B7 had no impact on the protein-protein interaction. These results provide valuable insights for a comprehensive understanding of UGT1A9/UGT2B7 heterodimer formation and its association with glucuronidation activity.
Significance Statement:

Our findings revealed that the H35A mutation in UGT2B7 affected the affinity of protein-protein interaction, leading to discernable variations in FRET efficiencies and catalytic activity. Furthermore, the signal peptide and single transmembrane helix domain of UGT2B7 did not influence heterodimer formation. These results provide valuable insights into the combined effects of polymorphisms and protein-protein interactions on the catalytic activity of UGT1A9 and UGT2B7 in vitro, enhancing our understanding of UGT dimerization and its impact on metabolite formation.
Introduction

Human UDP-glucuronosyltransferases (UGTs) serve as essential metabolic enzymes that transfer the glucuronic acid from UDP-glucuronic acid (UDPGA) to both endobiotic and xenobiotic compounds. (Meech and MacKenzie, 1998; Tukey and Strassburg, 2000). UGTs represent a category of type I transmembrane glycoproteins predominantly localized in the endoplasmic reticulum (ER) membrane. Comprising approximately 530 amino acids (Guillemette et al., 2010), UGTs encompass two distinct domains: the N-terminal domain (NTD), which incorporates a cleavable signal peptide (SP) responsible for ER membrane localization, and the C-terminal domain (CTD), which firmly tethers the protein to the ER membrane (Fig 1) (Radominska-Pandya et al., 2005). The NTD encompasses a substrate binding domain that exhibits significant variability, while the CTD contains a conserved UDPGA binding domain (Ouzzine et al., 1999). The CTD is composed of a solitary transmembrane (TM) helix followed by approximately 20 residues facing the cytosol, constituting the cytosolic tail (CT). Moreover, the CTD is characterized by a di-lysine motif (DM) (KKXX/KXKXX), which functions as a retrieval signal for UGTs anchored to the ER (Miyauchi et al., 2019). UGT1A9 and UGT2B7, as two integral members of this enzyme superfamily, play essential roles in the metabolism of a wide range of endogenous substances and drugs. For instance, UGT1A9 is known to metabolize propofol, irinotecan, mycophenolic acid (Guo et al., 2013), and flavopiridol (Yueh et al., 2001; Villeneuve et al., 2003), while UGT2B7 metabolizes morphine (Coffman et al., 1997), zidovudine (AZT) (Uchaipichat et al., 2008), estriol (Lépine et al., 2004), carbamazepine (Staines et al., 2004), and androsterone (Turgeon et al., 2001).
UGTs often function in coordination with other key enzymes involved in drug metabolism (Ishii et al., 2010; Iyanagi, 2007). Numerous studies have demonstrated that UGTs can form homodimers or heterodimers, serving as dimers for monoglucuronide formation or tetramers for diglucuronide formation (Ikushiro et al., 1997; Kurkela et al., 2004). Co-immunoprecipitation (Co-IP) experiments (Lewis et al., 2011) and quantitative fluorescence resonance energy transfer (FRET) techniques (Yuan et al., 2015a) have been employed to detect UGT homo- and heterodimers. Notably, UGT1A1 has been identified to create complexes with other UGT1A proteins (Operaña and Tukey, 2007). Our research team has recently reported the capability of UGT2B7 to form dimers with UGT1A1 and UGT1A9 (Yuan et al., 2016). Moreover, accumulating evidence suggests that dimerization significantly affects the catalytic activity of individual enzymes within the dimeric system (Liu et al., 2016). UGT2B7 dimerization with UGT1A1 and UGT1A9 can selectively influence the generation of two morphine metabolites (ZZ Yang et al., 2017). Additionally, the propofol glucuronidation activity of UGT1A9 in human hepatocytes is directly influenced by the expression levels of UGT2B7 (Konopnicki et al., 2013).

To date, several polymorphisms in UGT2B7 genes have been identified. The UGT2B7*2 variant (802C>T, H268Y) has been reported to occur at a frequency of 9.2% in the Chinese population (Lin et al., 2005). This variant has the potential to improve the efficacy of epirubicin-based adjuvant chemotherapy in breast cancer patients (Parmar et al., 2011).

Protein glycosylation is recognized as a significant post-translational modification (PTM) that influences protein folding, stability, and activity (Chen et al., 2009). N-glycosylation sites at residues 68 and 315 of UGT2B7 have been reported (Nagaoka et al., 2012). The His35
residue within the predicted active site of UGT2B7 is conserved among human and plant enzymes (Miley et al., 2007). Mutation of His35 to alanine results in a catalytically inactive enzyme.

In this study, we co-expressed UGT2B7 allozymes WT, H35A, H268Y, and N68A/N315A with wild-type UGT1A9 in insect cells using the Bac-to-Bac expression system to investigate the UGT1A9-UGT2B7 dimerization mechanism. We employed quantitative FRET and Co-IP techniques to assess heterodimerization. Furthermore, we examined the glucuronidation activities of the heterodimers using zidovudine as a substrate for UGT2B7 and propofol as a substrate for UGT1A9. Our objective is to elucidate the relationship between protein-protein interactions and glucuronidation activity of UGT1A9 and UGT2B7.
Materials and methods

Materials

Zidovudine, propofol, UDPGA, loratadine, dimethylsulfoxide (DMSO), iodoacetamide, and alamethicin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propofol O-glucuronide and zidovudine O-glucuronide were purchased from Toronto Research Chemicals (Toronto, Canada). The rabbit anti-UGT2B7 Polyclonal antibody was purchased from Abcam. Mouse anti-HA tag Monoclonal antibody was purchased from ProteinTech. High-purity acetonitrile was sourced from Merck (Darmstadt, Germany).

Constructs

The sequence of the human UGT2B7 (UniProt ID P16662) was cloned into the pFastBac1 vector. For the FRET and Co-IP study, a cyan fluorescent protein (CFP) and a 10 × His-tag were fused to the C-terminus of UGT2B7 (UGT2B7-CFP-His10). Four truncations (ΔN1-23, ΔC493-529, ΔC511-529, and ΔC525-529) and three mutations (H35A, H268Y, and N68A/N315A) were incorporated into UGT2B7. The wild-type human UGT1A9 sequence (UniProt ID O60656) was integrated into the pFastBac1 vector along with a yellow fluorescent protein (YFP) (UGT1A9-YFP) to facilitate the FRET investigation.

The UGTs exhibit comparable protein molecular weights, ranging from approximately 52 to 58 kDa. In the construction of Co-IP experiment constructs, the C-terminal CFP tag, previously introduced in UGT2B7 for FRET experiments, was retained to distinguish UGT2B7 from UGT1A9. Furthermore, a 10 × His-tag was added (UGT2B7-CFP-His10) for affinity chromatography using His-tag resins. At the C-terminus of wild-type UGT1A9, an
haemagglutinin (HA) tag was added (UGT1A9-HA) to facilitate protein expression and enable western blot analysis. Furthermore, the constructs employed in the Co-IP experiments were chosen to be consistent with those used in the enzyme activity assays.

**Expression of UGT1A9 and UGT2B7**

The heterologous expression of UGT1A9 and UGT2B7 in *Spodoptera frugiperda* (*Sf*9) insect cells was performed according to established protocols with slight modifications (Xue *et al.*, 2022). Briefly, the UGT1A9(WT) and UGT2B7*N genes, cloned into the pFastBac1 vector, were transformed into *Escherichia coli* DH10Bac cells, which were developed in-house for the generation of recombinant bacmid. For transfection, insect cells were diluted to a density of 1.1 × 10^6^ cells/mL and seeded into 24-well plates, with each well containing 2.5 mL of the cell suspension. The cells were then incubated at 27°C under agitation at 300 rpm for 96 hours to facilitate the generation of P0 viral stock. Subsequently, 40 mL of *Sf*9 cells with a density of 2.1 × 10^6^ cells/mL were mixed with 400 μL of P0 viral stock. The cells were incubated at 27°C with agitation at 125 rpm for 48 hours to generate a high-titer P1 viral stock. During the expression of UGT1A9(WT) and UGT2B7*N monomers, the density of *Sf*9 cells was adjusted to 2.5 × 10^6^ cells/mL, followed by the addition of 200 μL of P1 viral stock. For the expression of UGT1A9(WT)/UGT2B7*N heterodimers, the P1 viral stock of both proteins were introduced to the cells in a 1:1 ratio. After a 48-hour incubation, cells were harvested and stored at -80°C.

**FRET analysis**
Sf9 cells were seeded on 6-well plates at a density of 1.1 × 10^6 cells/well and co-infected with UGT1A9-YFP baculovirus and recombinant UGT2B7*N-CFP baculovirus. After 96 hours post-infection at 27°C, cells were washed and collected in 1 × PBS. Subsequently, the Sf9 cells were mixed with anti-fade mounting medium and placed onto slides. The FRET intensity was then captured using an Olympus BX61 confocal microscope (Olympus, Tokyo, Japan).

To determine FRET efficiency (E), the acceptor photobleaching method was applied, as described previously (Qin et al., 2022). Briefly, co-expressed Sf9 cells slides were imaged in the CFP channel (excitation: 405 nm, emitter: 476 nm) and the YFP channel (excitation: 515 nm, emitter: 527 nm). FRET efficiency (E) was calculated by the equation:

$$E = \frac{D_a - D_b}{D_a}$$

where $D_a$ is the average CFP fluorescence intensity after acceptor photobleaching, and $D_b$ is the average CFP fluorescence intensity before acceptor photobleaching. The distance (r) between the UGT2B7*N-CFP and UGT1A9-YFP was computed using the following equation:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

where $R_0$ represents the Förster distance (52.16 Å, manufacture data).

**Co-immunoprecipitation**

40 mL of Sf9 cell were initially isolated in a hypotonic buffer containing 10 mM HEPES, pH 7.5, 10 mM MgCl2, 20 mM KCl, and protease inhibitor cocktail (Rocha) using a dounce homogenizer. After ultra-centrifugation at 580,000 g for 30 minutes, the pelleted membranes were resuspended in a hypertonic buffer containing 10 mM HEPES, pH 7.5, 1.0 M NaCl, 10 mM MgCl2, 20 mM KCl, and protease inhibitor cocktail. Subsequently, the membranes were
resuspended in the presence of 2 mg/mL iodoacetamide and incubated at 4°C for 30 minutes before solubilizing in a solubilization buffer containing 50 mM HEPES, pH 7.5, 20 mM imidazole, 20% (v/v) glycerol, 800 mM NaCl, 0.5% (w/v) lauryl maltose neopentyl glycol (LMNG, Antrac), and 0.05% (w/v) cholesteryl hemisuccinate tris salt (CHS, Antrac). After 4 hours of solubilization at 4°C, UGT1A9, UGT2B7, and UGT1A9/UGT2B7*N were separated by ultra-centrifugation at 58,000 g for 1 hour. The supernatant was then incubated with the His-tag resin (TaKaRa) at 4°C overnight. On the following day, the resin was washed with 20 column volumes (CV) of wash buffer 1 (50 mM HEPES, pH 7.5, 30 mM imidazole, 400 mM NaCl, and 0.1/0.01% (w/v) LMNG/CHS), followed 15 CV wash buffer 2 (20 mM HEPES, pH 7.5, 45 mM imidazole, 200 mM NaCl, and 0.01/0.001% (w/v) LMNG/CHS). The target proteins were eluted with 6 CV elution buffer (20 mM HEPES, pH 7.5, 300 mM imidazole, 150 mM NaCl, and 0.002/0.0002% (w/v) LMNG/CHS). Eluted monomers or heterodimers were concentrated using a 50 kDa molecular weight cutoff (MWCO) concentrator. Samples were further detected by western blotting using anti-UGT2B7 and anti-HA antibodies and quantified with optical methods using the Image J software (Image J 1.5, NIH, USA).

**Enzyme activity assay**

To assess the glucuronidation capacity of monomers and heterodimers, we utilized AZT and propofol glucuronide formation to determine UGT2B7 and UGT1A9 metabolic activity. Initially, we weighed 13.4 mg of powdered AZT and 8.9 mg of powdered propofol, dissolving each in 100 μL of DMSO to create 500 mM stock solutions. Then, 40 mL of S/9 cells
expressing either UGT1A9 or UGT2B7 individually, as well as UGT1A9/UGT1B7*N dually expressed cells, underwent lysis using 2.5 mL of assay buffer composed of 100 mM potassium phosphate (pH 7.4), 10 mM MgCl2, and 0.05 mg/mL alamethicin. For AZTG formation, various concentrations of AZT (0, 0.05, 0.1, 0.2, 0.25, 0.5, 1, 1.25, 2.5, 5 mM) were added to incubation mixtures (Supp. Table 1). Each concentration of incubation solution was divided into three aliquots, each containing 45 μL. After preincubation at 37℃ for 5 minutes, 5 μL UDPGA (final concentration of 5 mM) was placed in mixtures to initiate reactions, resulting in a final incubation volume of 50 μL. After a 120-minute incubation period, the reactions were halted by introducing 100 μL of a quenching solution, composed of ice-cold acetonitrile containing loratadine as the internal standard (final concentration of 5 ng/mL). Insoluble materials in mixtures were removed by centrifugation at 12,000 g and 4℃ for 10 minutes, and the supernatant was stored at -20℃. For the propofol glucuronidation assay, the incubation system was similar to AZT (Supp. Table 1), but the concentration of the internal standard was increased to 100 ng/mL.

An Agilent 1290 liquid chromatography system coupled with a 6460 triple-quadrupole mass spectrometry (Agilent Technologies, Santa Clara, CA) was employed to quantify AZTG and PG. The samples (10 μL) were injected into a ZORBAX XDB column (3.5 μm, 50 mm × 2.1 mm) and eluted with a mobile phase comprising 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was set at 0.2 mL/min. For AZTG, the gradient elution program was as follows: 10%B at 0 - 1 minutes, 10% - 40%B at 1 – 1.5 minutes, 40%B at 1.5 – 4 minutes, 40% - 90%B at 4 – 4.5 minutes, and 90% - 10%B at 4.5 – 6 minutes. For PG, the gradient elution program was as follows: 20%B at 0 - 1
minutes, 20% - 60%B at 1 – 1.5 minutes, 60% - 90%B at 1.5 – 3 minutes, 90%B at 3 – 4 minutes, and 90% - 20%B at 4 – 5 minutes. The specific parameters for AZTG, PG, and the internal standard are presented in Table 1 (Chen et al., 2018; Verma and Singh, 2016).

Kinetic analysis was performed using GraphPad Prism software (version 8.0). The maximum velocity ($V_{\text{max}}$) and the Michaelis-Menten constant $K_m$ were calculated using the Michaelis-Menten equation:

$$V = \frac{V_{\text{max}} \times S}{K_m + S}$$

where $V$ represents the reaction velocity and $S$ is the substrate concentration. The intrinsic clearance ($CL_{\text{int}}$) was calculated by the equation:

$$CL_{\text{int}} = \frac{V_{\text{max}}}{K_m}$$

**Statistical analysis**

The values are presented as means ± standard deviation (SD) from three independent experiments with separate preparations. Statistical comparisons between different groups were performed by one-way ANOVA with a Dunnett’s posthoc test (SPSS 13.0 software, Chicago, IL, USA). A $P$ value < 0.05 was considered statistically significant.
Result

FRET analysis

The process of heterodimerization between UGT1A9 and UGT2B7N was investigated using FRET, a reliable method for evaluating protein-protein interactions. YFP-tagged UGT1A9 and CFP-tagged UGT2B7*N (WT, H35A, H268Y, N68A/N315A, ΔN1-23, ΔC1493-529, ΔC511-529, and ΔC525-529) enzymes were stably expressed in a Bac-to-Bac insect expression system, and visualized individually using fluorescence microscopy in both the YFP and CFP channels. The strength of the interaction between the two protein isoforms was quantitatively assessed by measuring the donor-acceptor distance (r) and FRET efficiency (%) (Table 2). A shorter donor-acceptor distance and higher FRET efficiency indicate a more robust interaction with the target protein (Algar et al., 2019).

The impact of specific mutations on protein conformation has been well-documented, leading to alterations in the substrate affinity of metabolic enzymes (Seppen et al., 1996; Xiong et al., 2008). As illustrated in Fig. 2, a notable increase in fluorescence intensity in the CFP channel was observed for UGT1A9(WT)-YFP/2B7(WT)-CFP compared to the CFP-linker-YFP (positive control) (Fig 2A, Supp. Fig. 1). These findings indicate the close proximity and formation of protein-protein interactions between UGT1A9 and UGT2B7. Furthermore, CFP-tagged UGT2B7 allozymes (H35A, H268Y, and N68A/N315A) were capable of forming heterodimers with wild-type UGT1A9 within the ER, where different donor-acceptor distances directly indicated diverse dimeric affinities. Upon comparing the acquired values of donor-acceptor distance (r) and FRET efficiency (%), it was evident that wild-type UGT2B7 exhibited the highest affinity towards UGT1A9, while UGT2B7(H35A) displayed the lowest
affinity.

To investigate the impact of the N- and C-terminal regions of UGT2B7 on heterodimer formation, a series of deletion mutants were designed. These mutants included UGT2B7 variants lacking the signal peptide (ΔN1-23), single transmembrane helix (ΔC493-529), cytosolic tail (ΔC511-529), and di-lysine motif (ΔC525-529). Comparing the obtained $E\%$ and $r$ values (Fig 3), it was observed that the deletion mutants lacking the signal peptide and single transmembrane helix of UGT2B7 exhibited a reduced affinity towards wild-type UGT1A9. Conversely, truncation of the cytosolic tail and the di-lysine motif resulted in minimal differences in FRET efficiency compared to the wild-type dimers.

**Co-immunoprecipitation**

In addition to FRET analysis, Co-IP techniques were employed to investigate the protein-level interactions between heterodimers. Negative controls were established to verify the normal expression of proteins and the experimental specificity. This was achieved by individually expressing constructs containing UGT1A9-HA fused with a 10 × His-tag and wild-type UGT2B7-CFP-His$_{10}$ in $Sf$9 cells. The solubilized whole proteins were then mixed with His-tag resins, and the purified complexes were subjected to individual detection by western blotting using anti-UGT2B7 and anti-HA antibodies. Upon interaction between UGT2B7 and UGT1A9, the Western blot analysis revealed the presence of two distinct bands in the immunoblot. These bands corresponded to UGT2B7*N-CFP, with an approximate molecular weight of 85 kDa, and UGT1A9-HA, with an approximate molecular weight of 58 kDa.

As a result (Fig. 4), when UGT2B7-CFP-His$_{10}$ allozyme and UGT1A9-HA-His$_{10}$ allozyme
were individually expressed, no co-immunoprecipitation was detected. However, upon co-expression of HA-tagged UGT1A9 and His-tagged UGT2B7 in insect cells, the presence of two bands was observed, indicating the immunoprecipitation of UGT2B7 with UGT1A9. Remarkably, compared to the UGT2B7(WT)/1A9(WT) heterodimer, the dimerization of UGT2B7(ΔN1-23) and UGT2B7(ΔC493-529) with UGT1A9 exhibited a significant decrease in affinity, respectively. Conversely, the mutants H35A, H268Y, and N68A/N315A, as well as the deletion mutants of the cytosolic tail and di-lysine motif, had no impact on the proportion of UGT2B7 in heterodimers. Taken together, these findings suggest that the amino acid sequence within the signal peptide or the single transmembrane helix influences the affinity of the UGT2B7/1A9 dimer. These results provide additional support to the quantitative FRET analysis.

**Catalytic analysis of UGT heterodimers**

To evaluate the influence of protein-protein interactions on the enzymatic activity of UGT1A9 and UGT2B7, we examined the glucuronidation activities of propofol and zidovudine in both single and double expression systems. The presence of the fusion protein CFP, as well as the HA and His tags added at the C-terminus, did not demonstrate any observable impact on the metabolic activities of UGT2B7 and UGT1A9 (Yuan et al., 2016; Zhang et al., 2012). Therefore, we utilized the same constructs and expression methodologies as employed in the Co-IP experiments. We utilized a modified LC-MS/MS method to quantify the rates of zidovudine O-glucuronide (AZTG) and propofol O-glucuronide (PG) formation by the heterodimers expressed in Sf9 cells (Supp. Fig. 2 and 3). The observed data exhibited a good
Kinetic analysis of AZTG formation

The kinetic parameters of wild-type UGT2B7 monomer, tagged with CFP, revealed a $K_m$ value of 1.54±0.04 mM and a $V_{max}$ value of 2.54±0.03 nmol/min/unit (Table 3). These $K_m$ and $V_{max}$ values are consistent with those reported in previous studies (Badée et al., 2019b; Horspool et al., 2020) that investigated the kinetic constants of UGT2B7 without a CFP tag. Regarding the $K_m$ value (Fig 5B and E), truncation of the signal peptide (ΔN1-23) or the transmembrane helix (ΔC493-529) resulted in the complete absence of detectable glucuronidation product AZTG generation in the incubation solution, suggesting a significant loss of UGT2B7's ability to glucuronidate AZT. Conversely, truncation of the di-lysine motif (ΔC525-529) led to a decrease in the $K_m$ value, indicating an enhanced affinity of UGT2B7 for AZT. Furthermore, all UGT2B7 mutants and truncations inhibited the velocities of metabolites (Fig 5C and F). The corresponding kinetic constants are provided in Table 3.

Kinetic analysis of PG formation

The impact of protein-protein interactions on the enzymatic properties of UGT1A9 was assessed using both single and double expression models. Propofol glucuronidation activity was measured based on the peak area of PG. Interactions with UGT2B7-CFP resulted in enhanced $V_{max}$ values for PG formation compared to UGT1A9 single-expressed enzymes (Fig 6B and C), indicating higher yields of UGT2B7(WT)-CFP/1A9(WT) heterodimers compared to UGT1A9(WT) single expression systems. When UGT1A9 was co-expressed with
UGT2B7(H35A), UGT2B7(ΔC_{511-529}), and UGT2B7(ΔC_{525-529}), it exhibited a decrease in the $K_m$ value for propofol metabolism compared to the wild-type heterodimer, suggesting an increased affinity of UGT1A9 towards propofol. Additionally, co-expression of UGT1A9 and UGT2B7(ΔN_{1-23}) significantly increased the $V_{max}$ values for PG formation (**Fig 6E and F**).

On the other hand, other mutants of UGT2B7 (H35A, H268Y, N68A/N351A, ΔC_{493-529}, ΔC_{511-529}, or ΔC_{525-529}) displayed a significant reduction in the $V_{max}$ value. The corresponding kinetic constants are detailed in **Table 4**. The formation of a heterodimer between UGT1A9 and UGT2B7 had a minimal impact on the $K_m$ value for propofol metabolism, whereas there was a substantial change in the $V_{max}$ value. This suggests that different UGT2B7 mutant and truncated forms can significantly alter the abundance of UGT1A9 in the system.
**Discussion**

UGT2B7 is primarily known to catalyze glucuronidation reactions as a monomer. However, its metabolic capacity is often altered when it forms homodimers or heterodimers (Fujiwara et al., 2016). Previous studies have demonstrated that UGT2B7 can form heterodimers with UGT1A9 (Yuan et al., 2015b). Limited by the intricate physiological environment they operate in, studying UGTs dimerization phenomena in vivo have proven challenging, impeding our understanding of the catalytic mechanisms of this enzyme family.

In this study, we utilized a baculovirus-insect cell expression system to overexpress the target proteins, focusing on UGT1A9 and UGT2B7 recombinant allozymes. Our main objective was to explore how dimerization influences the enzymatic activity of these two UGT isoforms. We employed FRET and Co-IP techniques to investigate the interactions between UGT1A9 and UGT2B7 allozymes during heterodimer formation, with the aim of uncovering potential mechanisms behind this dimerization process. Additionally, using the LC-MS/MS technique, we conducted a comprehensive analysis to examine how the formation of heterodimers impacts the glucuronidation activity of UGT1A9 and UGT2B7 toward their respective probe substrates, propofol and zidovudine. Notably, our study provides the first evidence that UGT2B7 allozymes, including H35A, N68A/N351A, ΔN1-23, ΔC493-529, ΔC511-529, and ΔC525-529, can form heterodimers with UGT1A9.

By aligning the amino acid sequences of glycosyltransferases (GTs) from human and plant, we observed a highly conserved histidine residue at position 35. Further, by integrating the three-dimensional structures of two plant GTs, flavonoid 3-O-glycosyltransferase (PDB ID: 2C1X) (Offen et al., 2006) and UGT71G1 (PDB ID: 2ACV) (Shao et al., 2005), we
elucidated the critical role of the histidine residue at position 35 in mediating the enzyme-substrate interaction. In this study, the UGT1A9/2B7(H35A) heterodimer exhibited significantly lower $E\%$ and longer $r$ compared to the wild-type heterodimer, without affecting the interaction ratio of the dimer. Furthermore, this mutation notably reduced the metabolic capacity of UGT2B7 towards AZT and altered the affinity of UGT1A9 for propofol. These observations suggest that the H35A mutation induce conformational changes in UGT2B7, leading to an increased distance between the dimeric proteins and attenuating their interaction. Additionally, the UGT2B7 mutants, single nucleotide polymorphisms (SNPs) H268Y and N-glycosylation N68A/N315A, exhibited UGT1A9 affinity comparable to that of the wild-type UGT2B7.

Truncation of the signal peptide or the transmembrane helix in UGT2B7 significantly affected the stability of the UGT1A9/UGT2B7 heterodimer. However, our findings demonstrate distinct roles for these two amino acid sequences in dimerization. The signal peptide of UGT2B7 has been reported that plays a key role in targeting the ER membrane and promoting proper protein folding (Troberg and Finel, 2015). In this study, deletion of the signal peptide profound effects on the FRET efficiency, protein ratio, and enzyme activity of the dimer. Firstly, truncation of the signal peptide resulted in an increase of over 2.5 nm in the distance between UGT2B7 and UGT1A9. Co-IP results indicated that the two proteins failed to properly interact and form a stable heterodimer. Additionally, enzyme activity assays revealed a substantial loss of UGT2B7's ability to metabolize AZT due to the truncation of the signal peptide. Under these conditions, the expressed UGT2B7 likely consisted of misfolded and inactive proteins, creating ample space in Sf9 cell membrane for the expression of UGT1A9.
monomeric proteins. This indirectly enhanced the rate of propofol metabolism by UGT1A9. Similarly, truncation of the transmembrane helix of UGT2B7 also weakened the dimeric interaction. The single transmembrane helix of UGT2B7 primarily anchors the enzyme to the ER membrane, ensuring protein conformation stability and facilitating proper glucuronidation reactions (Meech and Mackenzie, 1997; N Yang et al., 2017). Truncation of residues 493-525 led to an increased distance between UGT2B7 and UGT1A9, resulting in a significant loss of UGT2B7's ability to metabolize AZT, albeit to a lesser extent compared to the signal peptide truncation. Interestingly, the deletion of the transmembrane helix in UGT2B7 impacted the protein ratio of UGT1A9, consequently altering the rate of propofol metabolism. It is known that UGTs require the presence of the ER membrane for optimal glucuronidation activity (Kurkela et al., 2003). Therefore, the absence of the transmembrane helix weakens the interaction between UGT2B7 and the ER membrane, leading to an unstable fit of the ER lumen region against the membrane. This dynamic conformational state hinders the catalytic transfer of glucuronide groups and impedes the expression of UGT1A9 in the system, ultimately resulting in a decrease in the interaction force between the heterodimers.

Deletion of the di-lysine motif or substitution of lysines in the motif for alanines has been reported that severely impairs the 4-methylumbelliferone glucuronidation activity of UGT1A9 (Miyauchi et al., 2020). Our results confirmed that the deletion of DM also affected the propofol glucuronidation activity of UGT1A9.

In conclusion, the findings presented in this manuscript provide support for the dimerization of UGT1A9 and UGT2B7 allozymes through FRET and Co-IP analyses. The H35A mutation in UGT2B7 influenced the affinity of protein-protein interaction, leading to distinct FRET
efficiencies and catalytic activity. Interestingly, deletion of the signal peptide and transmembrane helix in UGT2B7 altered AZT glucuronidation without affecting propofol glucuronidation, indicating that these regions of UGT2B7 did not impact heterodimer formation. These results offer valuable insights into the combined effects of polymorphisms and protein–protein interactions on the catalytic activity of UGT1A9 and UGT2B7 in vitro, enhancing our understanding of UGT dimerization and its impact on metabolite formation.
Acknowledgments

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions

Participated in research design: Jia Xue, Su Zeng, Haitao Zhang

Conducted experiments: Jia Xue, Jing Nie

Performed data analysis: Jia Xue, Jiayi Yin

Wrote or contributed to the writing of the manuscript: Jia Xue, Huidi Jiang, Su Zeng
Reference


Kurkela M, Garcia-Horsmant JA, Luukkanen L, Mörsky S, Taskinen J, Baumann M, Kostiainen R, Hirvonen J, and Finel M (2003) Expression and characterization of recombinant human UDP-glucuronosyltransferases (UGTs): UGT1A9 is more resistant to detergent inhibition than the other UGTs and was purified as an active dimeric


Physiol.


Yuan L, Qian S, Xiao Y, Sun H, and Zeng S (2015a) Homo- and hetero-dimerization of human UDP-glucuronosyltransferase 2B7 (UGT2B7) wild type and its allelic variants


Footnotes

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No author has an actual or perceived conflict of interest with the contents of this article.

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Figure Legends

Fig 1. Schematic representation of the UGT1A9 (A) and UGT2B7 (B) protein primary structures derived from each locus.

Fig 2. Detection of fluorescence intensity subsequent to the infection of Sf9 cells with recombinant UGT2B7*N-CFP and UGT1A9-YFP baculoviruses. Sf9 cells co-infected with the following recombinant constructs: (A) UGT2B7(WT)-CFP+UGT1A9(WT)-YFP, (B) UGT2B7(H35A)-CFP+UGT1A9(WT)-YFP, (C) UGT2B7(H268Y)-CFP+UGT1A9(WT)-YFP, and (D) UGT2B7(N68A/N315A)-CFP + UGT1A9(WT)-YFP. An increase in CFP fluorescence intensity was observed in the arrow areas bleached by the 515 nm laser line. The FRET efficiency was compared in (E). Notably, the H35A mutant exhibited a significant reduction in FRET efficiency between UGT1A9 and UGT2B7 compared to the wild-type UGT2B7. The asterisks indicate statistically significant differences when comparing the heterodimer UGT2B7(H35A)/1A9(WT) to UGT2B7(WT)/1A9(WT) (****P < 0.0001).

Fig 3. FRET phenomenon detected by acceptor photobleaching and calculated FRET efficiency of UGT2B7 deletion mutants with wild-type UGT1A9. Sf9 cells were co-infected with the following recombinant constructs: (A) UGT2B7(ΔN1-23)-CFP+UGT1A9-YFP, (B) UGT2B7(ΔC493-529)-CFP+UGT1A9-YFP, (C) UGT2B7(ΔC511-529)-CFP+UGT1A9-YFP, and (D) UGT2B7(ΔC525-529)-CFP+UGT1A9-YFP. An increase in CFP fluorescence was observed in the arrow areas bleached by the 515 nm laser line. The FRET efficiency was compared in (E). Truncation of the signal peptide (ΔN1-23) and the transmembrane helix (ΔC493-529) resulted
in a significant reduction in FRET efficiency between UGT1A9 and UGT2B7 compared to the wild-type UGT2B7. The asterisks indicate statistically significant differences when comparing the heterodimers UGT2B7(ΔN1-23)/1A9(WT) and UGT2B7(ΔC493-529)/1A9(WT) to UGT2B7(WT)/1A9(WT) (**P < 0.0001).

**Fig 4.** Analysis of the dimerization of UGT1A9 and UGT2B7 allozymes by Co-IP. (A) Color-coded domain architecture of human UGT2B7, with residue positions of the wild-type UGT2B7 indicated by numbers counted from the N-terminus. The dashed line represents the truncation. (B) Sf9 cell lysates from individually or co-expressed UGT1A9 and UGT2B7 allozymes were immunoprecipitated with anti-His resins, followed by western blot analysis using anti-UGT2B7 and anti-HA antibodies. (C) Proportion of UGT2B7 in heterodimers. The intensities of UGT2B7*N and UGT1A9 bands within each group were divided and compared to the wild-type heterodimer, resulting in the proportion of UGT2B7*N. The asterisks indicate statistically significant differences in the ratio of UGT2B7 in dimers when comparing the heterodimers UGT2B7(ΔN1-23)/1A9(WT) and UGT2B7(ΔC493-529)/1A9(WT) to UGT2B7(WT)/1A9(WT) (**P < 0.0001).

**Fig 5.** Kinetic analysis of AZTG formation by the single and double expression system. (A, D) Michaelis–Menten plots of AZTG in the UGT2B7-CFP single expression system and UGT1A9/UGT2B7*N (WT, H35A, H268Y, N68A/N351A, ΔN1-23, ΔC493-529, ΔC511-529, or ΔC525-529) double expression system. Comparison of $K_m$ values (B, E) and $V_{max}$ values (C, F) of heterodimers. Significant differences in $K_m$ and $V_{max}$ values for AZTG formation were
observed among the different groups compared to the UGT1A9(WT)/UGT2B7(WT)-CFP heterodimers. The asterisks indicate statistically significant differences (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.005$).

**Fig 6.** Kinetic analysis of PG formation by the single and double expression system. **(A, D)** Michaelis–Menten plots of PG in the UGT1A9(WT) single expression system and UGT1A9/UGT2B7*N (WT, H35A, H268Y, N68A/N351A, ΔN1-23, ΔC493-529, ΔC511-529, or ΔC525-529) double expression system. Comparison of $K_m$ values **(B, E)** and $V_{max}$ values **(C, F)** of heterodimers. Compared to the UGT1A9(WT)/UGT2B7(WT)-CFP heterodimers, there were significant differences in the $K_m$ and $V_{max}$ values for PG formation among the different groups. The asterisks indicate statistically significant differences (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.005$, * $P < 0.05$).
Table 1. Analytical parameters for the metabolites and internal standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM</th>
<th>Transition (m/z)</th>
<th>Fragment (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zidovudine β-D-glucuronide</td>
<td>Negative</td>
<td>442.3/125.0</td>
<td>150</td>
<td>22</td>
</tr>
<tr>
<td>Propofol β-D-glucuronide</td>
<td>Negative</td>
<td>353.2/177.2</td>
<td>230</td>
<td>24</td>
</tr>
<tr>
<td>Loratadine (IS)</td>
<td>Positive</td>
<td>383.1/337.1</td>
<td>170</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 2. FRET efficiency \((E\%)\) and donor-acceptor distances \(r\).

<table>
<thead>
<tr>
<th>protein-protein</th>
<th>FRET efficiency (E%)</th>
<th>Donor-acceptor distance (r) (nm)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP-linker-YFP</td>
<td>19.2±4.50</td>
<td>6.66</td>
<td>10</td>
</tr>
<tr>
<td>1A9-YFP/2B7(WT)-CFP</td>
<td>43.6±6.10</td>
<td>5.45</td>
<td>16</td>
</tr>
<tr>
<td>1A9-YFP/2B7(H35A)-CFP</td>
<td>32.4±6.10</td>
<td>5.92</td>
<td>11</td>
</tr>
<tr>
<td>1A9-YFP/2B7(H268Y)-CFP</td>
<td>39.8±5.00</td>
<td>5.69</td>
<td>9</td>
</tr>
<tr>
<td>1A9-YFP/2B7(N68A/N315A)-CFP</td>
<td>38.2±4.75</td>
<td>5.66</td>
<td>10</td>
</tr>
<tr>
<td>1A9-YFP/2B7(ΔN1-23)-CFP</td>
<td>10.6±3.74</td>
<td>7.54</td>
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</tr>
<tr>
<td>1A9-YFP/2B7(ΔC493-529)-CFP</td>
<td>21.6±4.95</td>
<td>6.52</td>
<td>13</td>
</tr>
<tr>
<td>1A9-YFP/2B7(ΔC511-529)-CFP</td>
<td>44.4±4.33</td>
<td>5.43</td>
<td>11</td>
</tr>
<tr>
<td>1A9-YFP/2B7(ΔC525-529)-CFP</td>
<td>44.2±3.48</td>
<td>5.44</td>
<td>8</td>
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</tbody>
</table>

\(n\) is the sample size.
Table 3. Kinetic parameters for AZTG.

<table>
<thead>
<tr>
<th>UGT1A9/UGT2B7</th>
<th>$K_m$ (mmol/L)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$CL_{int}$ (μL/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B7(WT)-CFP monomer</td>
<td>1.54±0.04</td>
<td>2.54±0.03</td>
<td>1.65±0.03</td>
</tr>
<tr>
<td>1A9(WT)/2B7(WT)</td>
<td>0.97±0.11</td>
<td>1.02±0.04</td>
<td>1.06±0.07</td>
</tr>
<tr>
<td>1A9(WT)/2B7(WT)-CFP</td>
<td>1.08±0.12</td>
<td>1.38±0.10</td>
<td>1.29±0.05</td>
</tr>
<tr>
<td>1A9(WT)/2B7(H35A)-CFP</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1A9(WT)/2B7(H268Y)-CFP</td>
<td>0.94±0.02</td>
<td>0.47±0.01</td>
<td>0.50±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(N68A/N315A)-CFP</td>
<td>1.06±0.07</td>
<td>0.21±0.01</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔN1-23)-CFP</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔC493-529)-CFP</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔC511-529)-CFP</td>
<td>1.05±0.01</td>
<td>0.04±0.001</td>
<td>0.04±0.001</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔC525-529)-CFP</td>
<td>0.54±0.01</td>
<td>0.17±0.001</td>
<td>0.31±0.01</td>
</tr>
</tbody>
</table>

Data are mean ± SD of three independent determinations; N/A, not available.
Table 4. Kinetic parameters for PG.

<table>
<thead>
<tr>
<th>UGT1A9/UGT2B7</th>
<th>$K_m$ (mmol/L)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>$CL_{int}$ (nL/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A9(WT)</td>
<td>0.76±0.06</td>
<td>94.8±2.61</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(WT)</td>
<td>0.90±0.01</td>
<td>104±2.08</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(WT)-CFP</td>
<td>0.72±0.05</td>
<td>229±14.6</td>
<td>0.32±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(H35A)-CFP</td>
<td>0.51±0.09</td>
<td>171±5.65</td>
<td>0.34±0.05</td>
</tr>
<tr>
<td>1A9(WT)/2B7(H268Y)-CFP</td>
<td>0.86±0.14</td>
<td>120±7.21</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(N68A/N315A)-CFP</td>
<td>0.78±0.03</td>
<td>133±2.81</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔN1-23)-CFP</td>
<td>0.71±0.02</td>
<td>314±4.84</td>
<td>0.44±0.02</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔC493-529)-CFP</td>
<td>0.72±0.05</td>
<td>41.8±2.18</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔC511-529)-CFP</td>
<td>0.54±0.07</td>
<td>80.2±4.23</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>1A9(WT)/2B7(ΔC525-529)-CFP</td>
<td>0.54±0.03</td>
<td>177±5.23</td>
<td>0.33±0.02</td>
</tr>
</tbody>
</table>

Data are mean ± SD of three independent determinations.
Heterodimerization of human UDP-glucuronosyltransferase (UGT) 1A9 and UGT2B7 alters their glucuronidation activities

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\textsuperscript{d} The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310009, China.
Supplementary Fig 1. Fluorescence intensity analysis in S/9 cells infected with positive and negative control. (A) S/9 cells infected with CFP-linker-YFP baculovirus served as the positive control. S/9 cells infected with the following recombinant constructs: (B) UGT2B7(H35A)-CFP, (C) UGT2B7(H268Y)-CFP, (D) UGT2B7(N68A/N315A)-CFP, (E) UGT2B7(ΔN1-23)-CFP, (F) UGT2B7(ΔC493-529)-CFP, (G) UGT2B7(ΔC511-529)-CFP, and (H) UGT2B7(ΔC525-529)-CFP were used as the negative control. CFP fluorescence was observed in the areas marked by arrows, which were bleached by the 515 nm laser line.
**Supplementary Fig 2.** HPLC chromatograms of AZTG, PG, and the internal standard loradine. **(A)** From top to bottom are chromatograms of loradine (retention time 3.6 min) and AZTG (retention time 2.7 min). **(B)** From top to bottom are chromatograms of loradine (retention time 2.8 min) and PG (retention time 3.0 min).
Supplementary Fig 3. The standard curves of (A) AZTG and (B) PG. These curves were generated by plotting the reciprocal of fivefold AZTG (tenfold PG) standard concentrations in the incubation mixture against the peak area ratio of AAZTG (APG) to Aloratadine. The standard curve equation in (A) $y = 0.060146x - 0.003964$ ($R^2 = 0.999$) demonstrates a linear relationship between the peak area ratio and the reciprocal of AZTG concentrations within 0.01 to 5 mM. The standard curve equation in (B) $y = 0.667576x - 0.342806$ ($R^2 = 0.999$) demonstrates a strong linear relationship between the peak area ratio of PG to loratadine and the reciprocal of PG concentrations within the range of 5 - 500 μM.
Supplementary Table 1: Formulation of AZT/propofol incubation solution for enzyme activity analysis.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Stock solution (500 mM) (μL)</th>
<th>Mixture solution (μL)</th>
<th>Cell lysate (μL)</th>
<th>Final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1#</td>
<td>4</td>
<td>/</td>
<td>396</td>
<td>5</td>
</tr>
<tr>
<td>2#</td>
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<td>/</td>
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</tr>
<tr>
<td>5#</td>
<td>/</td>
<td>50 μL from Tube 2#</td>
<td>200</td>
<td>0.5</td>
</tr>
<tr>
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<td>/</td>
<td>50 μL from Tube 3#</td>
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