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Short Communication

The Polymorphic Variant P24T of UDP-Glucuronosyltransferase 1A4 and Its Unusual Consequences

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ABSTRACT

The P24T polymorphic variant of the human UDP-glucuronosyltransferase 1A4 (UGT1A4*2, 70C>A) occurs within the signal peptide, five amino acids upstream of the cleavage site and the start of the mature protein. Bioinformatic analysis of the variant suggested that the signal peptide of part of the translated protein is cleaved two residues upstream of the regular site, whereas the rest is cleaved as usual. To test this, recombinant UGT1A4-P24T, with a C-terminal His-tag, was expressed in sf9 insect cells and affinity-purified for N-terminal protein sequencing. The results were in agreement with the in silico prediction. About half of the mutant protein was cleaved at the regular site, between S28 and G29, whereas the other half was cleaved two amino acids upstream, between A26 and E27. The glucuronidation of two substrates, dexmedetomidine and trifluoperazine, was assayed using membrane-enriched UGT1A4-P24T and wild-type UGT1A4. The variant exhibited much lower glucuronidation rates, but kinetic analyses revealed large differences between them only in the V_{max} values. The K_m values for both substrates were not affected by the mutation and its consequences. This might suggest that the unusual signal peptide cleavage in UGT1A4-P24T somehow disturbs protein folding. Moreover, it raises the possibility that the effect of UGT1A4-P24T on the glucuronidation rate in mammalian expression systems would be mild since they contain more effective post-translation protein control systems in the endoplasmic reticulum. In summary, our results reveal the effect of a polymorphic mutation on the signal sequence cleavage and thereby also the mature UGT.

Introduction

UDP-glucuronosyltransferases (UGTs) (EC 2.4.1.17) catalyze the conjugation of xenobiotic or endobiotic substrates with glucuronic acid from the cofactor UDP-α-D-glucuronic acid (UDPGA). The glucuronidation mostly renders them biologically inactive and enhances their elimination via bile or urine (Tukey and Strassburg, 2000). The human UGT1A4 belongs to the UGT1 family (Mackenzie et al., 2005) and is mainly expressed in the liver, along with low expression in extrahepatic tissues (Court et al., 2012). Unlike most other UGT1 family members, which mostly catalyze the attachment of glucuronic acid to a substrate's hydroxyl group, UGT1A4 favors *N*-glucuronidation to primary, secondary, or tertiary amines (Green and Tephly 1996; Kaivosaari et al., 2011). This preference for *N*-glucuronidation reactions is probably due to the replacement of the "catalytic His" with a proline residue at position 40 of the protein precursor (Kubota et al., 2007). Since the UGT1A4 protein starts with a 28-amino acid–long signal peptide that directs the protein to the endoplasmic reticulum, the catalytic site is very close to the N-terminus of the mature protein, raising the possibility that any changes in accurate cleavage of the signal peptide will be reflected in the enzymatic activity.

Several different genetic variations or polymorphisms in UGT genes were found, and their influence has been studied widely. Among the UGT1A4 variants, L48V was found to lead to higher glucuronidation rates, resulting in faster elimination of the epilepsy drug lamotrigine (Gulcebi et al., 2011). Another previously identified functional variant of UGT1A4 is P24T, which is rather common, with an allelic frequency of 8% in Caucasians, but the P24T variant is rarely homozygous (Ehmer et al., 2004; Lopez et al., 2013). The first studies with liver microsomes carrying the P24T variant used the major metabolite of a tobacco-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), as a substrate and reported a higher activity than the wild type (Wiener et al., 2004). Four years later, however, the same laboratory identified UGT2B10 as a major contributor to NNAL glucuronidation in microsomes (Chen et al., 2008). While the *N*-glucuronidation activity of UGT2B10 was not yet known in 2004, it now appears that the NNAL glucuronidation activity measured then in human liver microsomes carrying the P24T variant of UGT1A4 (Wiener et al., 2004) could have been due to activity of UGT2B10.

Studies with recombinant UGT1A4, expressed in HEK293 cells, yielded mixed results, ranging from a lower activity of the UGT1A4-P24T variant in comparison with the wild-type enzyme when the glucuronidation of two carcinogenic amines and two endogenic steroids were tested (Ehmer et al., 2004) to no significant difference in the clearance of several other compounds (Sun et al., 2006; Edavana et al., 2013).

The P24T amino acid change takes place five residues before the cleavage site of the signal peptide and the starting point of the mature protein. A change in the amino acid sequence in this region may affect the cleavage of the signal peptide and hence the length and structure of the mature protein. After realizing the predicted effect of the P24T on the cleavage site of the signal peptide of UGT1A4 and that the modified residue itself is outside the mature protein (see Fig. 1), we have re-examined the effect of this mutation on activity. Wild-type UGT1A4

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ABBREVIATIONS: DME, dextromedetomidine; HPLC, high performance liquid chromatography; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; TFP, trifluoperazine; UDPGA, UDP-α-D-glucuronic acid; UGT, UDP-glucuronosyltransferase.

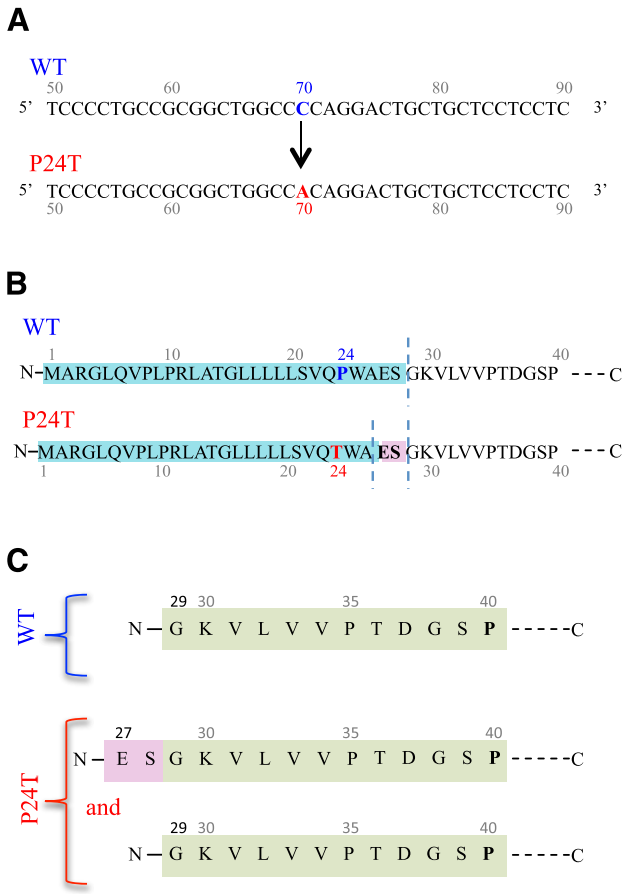

Fig. 1. The differences between UGT1A4 wild type (WT) and the UGT1A4-P24T variant (P24T) at the level of DNA (A), predicted cleavage site(s) of the signal peptide (B), and the N-termini identified following affinity purification and protein sequencing (C).

and the UGT1A4-P24T variant were expressed in insect cells for activity assays and purifications for N-terminal protein sequencing. The results demonstrated clearer and more significant effects of the mutation than previously reported, which might also give insights as to why the reported effect in mammalian microsomes is much milder.

Materials and Methods

Chemicals and Reagents. Dexmedetomidine (DME) was kindly provided by Orion Corporation Orion Pharma (Espoo, Finland). Trifluoperazine (TFP) dihydrochloride, UDPGA (ammonium salt), sodium phosphate monobasic dihydrate, and disodium hydrogen phosphate were purchased from Sigma (St. Louis, MO). Magnesium chloride hexahydrate and perchloric acid were from Merck (Darmstadt, Germany). Acetonitrile and methanol were liquid chromatography–mass spectrometry grade.

Recombinant UGT1A4 and UGT1A4-P24T Expression. Recombinant UGT1A4 with a C-terminal His-tag was prepared and expressed in baculovirus-infected sf9 insect cells, as previously described (Kurkela et al., 2003). The P24T variant was prepared from UGT1A4 by mutagenesis according to the QuikChange methodology (Stratagene, Jolla, CA) and the following mutagenesis oligonucleotides:

1. (Sense) 5′-CTCCTCAGTGTCCAGACCTGGGCTGAGAGTGGA-3′
2. (Antisense) 5′-TCCACTCTCAGCCCAGGTCTGGACACTGAGGAG-3′

The mutated gene was fully sequenced to verify that no additional mutation occurred and then was expressed in insect cells in the same way as the wild type UGT1A4. The C-terminal His-tag was used for expression level determination, as previously described (Kurkela et al., 2007). For glucuronidation activity assays, the expression levels of UGT1A4 and UGT1A4-P24T were optimized, and UGT-enriched membrane fractions were prepared as previously described (Zhang et al., 2012). The expression level of UGT1A4 was set as 1.0, whereas the relative expression level of UGT1A4-P24T was significantly higher at 5.1.

Recombinant UGT Purification for Protein Sequencing. Recombinant UGTs were purified from the UGT-enriched membranes using immobilized metal affinity chromatography on a 1-ml HiTrap Chelating HP column (Amersham GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using one column per purification. The column was preloaded with 0.5 ml of 100 mM $NiCl_2$ in water, washed, and pre-equilibrated with buffer A containing 20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 20 mM imidazole, and 0.2% SDS. The membranes, at 3 mg protein/ml, were extracted with 2% SDS in the presence of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 20 mM imidazole. After mixing, the suspensions were incubated at room temperature for 30 minutes, followed by 30-minute centrifugation at 40,000*g* at 20°C. The supernatant was loaded on the pre-equilibrated HiTrap Chelating HP column that was subsequently washed with buffer A described above. The bound protein was eluted using buffer B (buffer A supplemented with 400 mM imidazole), and fractions of about 0.2 ml were collected manually. The fractions were analyzed by SDS-PAGE, and a selected fraction was sent for N-terminal protein sequencing at the Proteomics Unit, Biotechnology Institute, University of Helsinki (http://www.biocenter.helsinki.fi/bi/protein/pro.html).

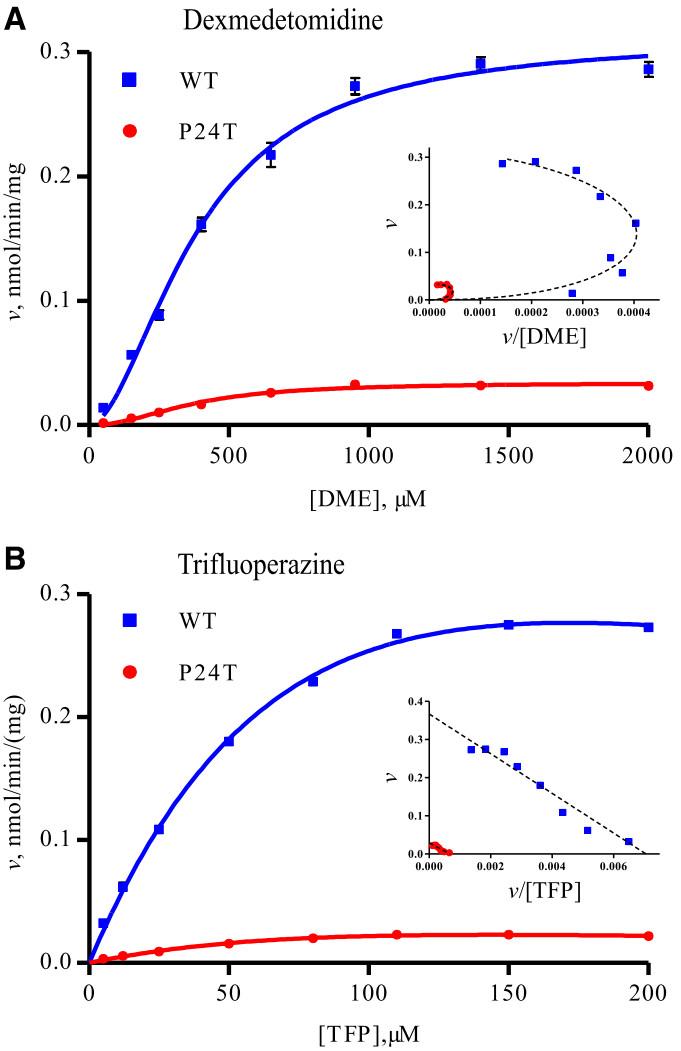

Fig. 2. Glucuronidation kinetics of dexmedetomidine (A) and trifluoperazine (B) by UGT1A4 and the variant UGT1A4-P24T. The derived kinetic constants are given in Table 1.

Glucuronidation Activity Kinetics. Appropriate concentrations of DME in methanol were transferred to tubes, the organic solvent was evaporated, and the substrates were dissolved in a reaction mixture containing 50 mM phosphate buffer, pH 7.4, 10 mM $MgCl_2$, and 0.2 mg/ml UGT-enriched membranes. The tubes were placed at +4°C for 15 minutes. In the case of TFP, the substrate stock was made in dimethylsulfoxide and, accordingly, the samples contained 1% dimethylsulfoxide. Subsequently, the tubes were preincubated for 5 minutes at 37°C, and the glucuronidation reaction was initiated by the addition of UDPGA, bringing the final concentration to 5 mM and the total volume to 100 μl.

The DME glucuronidation reactions were carried out for 60 minutes at 37°C and terminated by the addition of 60 μl of cold 1:5 4 M perchloric acid to methanol mixture. For TFP, the reactions were carried out for 30 minutes and terminated by the addition of 100 μl of cold 5% acetic acid in methanol. The tubes were transferred to −20°C for 15 minutes and centrifuged at 16,000*g* for 10 minutes, and then the supernatants were subjected to high-performance liquid chromatography (HPLC) analyses.

Analytical Methods. An Agilent 1100 equipped with a multiple wavelengths UV detector and Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm; Agilent Technologies, Palo Alto, CA) at a column temperature of +40°C was used for HPLC analyses. For the DME method, a gradient elution was done with phosphate buffer, pH 3.0, as eluent A and acetonitrile as eluent B: 0–7 minutes, 10→45% B; 7–7.1 minutes, 45→10% B, and 7.1–10 minutes, 10% B. For TFP, 0.1% formic acid was used as eluent A, whereas B was acetonitrile in the method 0–1 minutes, 35% B; 1–4 minutes, 35→80% B; 4–5 minutes, 80% B; 5–5.1 minutes, 80→35% B, and 5.1–7 minutes, 35% B. The flow rate was 1 ml/min, and the injection volume was 50 μl. The UV detection wavelengths were 215 nm (DME) and 259 nm (TFP). Glucuronide and parent compound retention times were 4.8 and 6.3 minutes for DME and 3.8 and 4.2 minutes for TFP, respectively. In the absence of glucuronide standards, we used the absorbance of the parent compounds for standard curve preparations, as previously recommended (Court 2005). The limits of detection and quantification for DME were 0.21 and 0.71 μM, whereas for TFP, they were 0.01 and 0.04 μM, respectively, calculated based on signal to noise ratios of 3 and 10. The substrate consumption was below 10% in all the samples. The protein concentrations and incubation times in the kinetic assays were within the linear range (suitable concentrations and times were selected in preliminary experiments). The assays were performed in triplicates, including a control without UDPGA and a control without substrate.

Analysis of Glucuronidation Kinetics. GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA) was used to calculate the kinetic values from HPLC data. The best kinetic model was selected by visual inspection of the Eadie-Hofstee plot and calculated r^2 value. The following models were chosen to calculate the kinetic parameters from the experimental data:

Michaelis-Menten model

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Sigmoidal model (Hill equation)

$$v = \frac{V_{max}[S]^h}{S_{50}^h + [S]^h}$$

where v is the initial velocity of the reaction, $[S]$ is the substrate concentration, V_{max} is the maximum reaction velocity, K_m or S_{50} is the concentration of the substrate at 0.5 of V_{max}, and h is the Hill coefficient.

Results and Discussion

We noticed that the location of P24 is within the signal peptide of UGT1A4 and wondered if it affects the signal peptide cleavage site. A bioinformatic analysis, using the SignalP 4.1 program (Petersen et al., 2011; http://www.cbs.dtu.dk/services/SignalP/), indicated that UGT1A4-P24T has a complex and rather unusual signal peptide cleavage. About half of the signal peptide is predicted to be cleaved correctly, between S28 and G29, whereas the other half is cleaved two residues upstream, between A26 and E27 (Fig. 1).

To better understand the effects on activity as well as the suitability of the SignalP prediction to the insect cell expression system, we have expressed UGT1A4 and UGT1A4-P24T as C-terminal His-tagged proteins, analyzed the activity of the enzymes, and purified them by affinity chromatography in the presence of SDS for N-terminal sequencing. The N-terminal sequencing results were in agreement with the SignalP prediction. Recombinant UGT1A4-P24T yielded both correctly cleaved UGT1A4 and longer by two residues UGT1A4 (Fig. 1). The activity assays revealed that the mixed signal peptide cleavage site sample, UGT1A4-P24T, had a large negative effect on the enzyme (Fig. 2), which is much larger than previously published. We have used two UGT1A4 substrates, DME and TFP, and the glucuronidation rate of UGT1A4-P24T of both these compounds, in comparison with wild-type UGT1A4, was rather similarly affected (Fig. 2).

Kinetic analyses revealed that the main difference between UGT1A4 and UGT1A4-P24T is in the V_{max} values (corrected for relative expression level), whereas the K_m and the kinetic models are the same (Table 1). This strongly suggests that the UGT1A4-P24T membranes contained a considerable amount of wrongly folded UGT1A4 that is inactive and may even inhibit the activity of the correctly folded enzyme by forming malfunctioning oligomers with it.

It may be interesting to speculate why the effect of the polymorphism on the signal peptide cleavage and hence on the protein folding or alteration of its post-translation modification is so high in the baculovirus-transfected insect cell expression system, which is clearly much higher than in HEK293 cells (Zhou et al., 2011) or human carriers (Reimers et al., 2014).

TABLE 1

Kinetic analysis data for UGT1A4 and variant UGT1A4-P24T (UGT1A4*2)

Enzyme kinetics constants are presented with standard errors and 95% confidence intervals in parentheses. The rates were corrected (normalized) for relative expression levels (see *Materials and Methods*), and the extra sum-of-squares (*F* test in GraphPad Prism) was used for the calculation of V_{max} differences as *P* values. The kinetic models that were used for the calculation of the constants are presented.

UGT Enzyme	K_m ± S.E. (95% Confidence Intervals)	V_{max} ± S.E. (95% Confidence Intervals)	Hill Coefficient, h ± S.E. (95% Confidence Intervals)	Model, r^2
	μM	*relative velocity units/total protein concentration*[a]		
Dexmedetomidine				
1A4	387.7 ± 17.4 (351.5–423.9)	312.0 ± 7.8 (295.8–328.1)	1.8 ± 0.1 (1.6–2.1)	HE, 0.99
1A4-P24T	371.0 ± 20.8 (327.6–414.3)	34.1 ± 1.0 (31.9–36.3)*	2.1 ± 0.2 (1.7–2.5)	HE, 0.98
Trifluoperazine				
1A4	52.0 ± 4.5 (42.7–61.2)	366.8 ± 11.3 (343.4–390.3)		MM, 0.99
1A4-P24T	42.7 ± 6.4 (29.4–55.9)	29.1 ± 1.4 (26.1–32.1)**		MM, 0.95

HE, Hill equation; MM, Michaelis-Menten equation.

[a]The rates were calculated as pmol glucuronide/min per mg total protein (using standard curves made with parent compounds' UV absorption maxima) and corrected for relative expression levels of recombinant UGT1A4 and UGT1A4-P24T for a more reliable comparison (see *Materials and Methods*).

*$P < 0.05$; **$P < 0.01$.

While most of the reported human carriers of UGT1A4*2 were heterozygous, a homozygous carrier was also found (Wiener et al., 2004, Reimers et al., 2014) and the transfected HEK293 cells carried no partner correct UGT1A4 allele. We suggest that the main reason for the difference is a (much) better system in the mammalian cells for the detection and removal of wrongly folded proteins in the endoplasmic reticulum, as previously reported (Polgar et al., 2006). This might suggest that the real difference in the effect of the mutation, once one understands the expression system, is not large, and the system described here is suitable for expressing, retaining, and affinity purifying the inactive variant alongside the wild type to examine whether the predicted effect of the mutation on the signal peptide cleavage site indeed occurred.

In summary, in this study we have examined the effect of a polymorphic mutation in UGT1A4 that takes place within the signal peptide. Bioinformatic prediction suggested that it leads to complex cleavage of the signal peptide and this was confirmed by N-terminal sequencing of purified protein. The change in the signal peptide cleavage site seemed to abolish enzymatic activity. We suggest that saturation of the insect cells' protein quality control system with the inactive UGT1A4-P24T is the main reason for the apparently more severe effect of the mutation in this expression system than in mammalian ones.

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Participated in research design: Troberg, Finel.
Conducted experiments: Troberg, Finel.
Performed data analysis: Troberg, Finel.
Wrote or contributed to the writing of the manuscript: Troberg, Finel.

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