Identification of Human UDP-Glucuronosyltransferase Isoform(s) Responsible for the Glucuronidation of 2-(4-Chlorophenyl)-5-(2-Furyl)-4-Oxazoleacetic Acid (TA-1801A)

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Summary: We characterized the hepatic and intestinal UDP-glucuronosyltransferase (UGT) isoform(s) responsible for the glucuronidation of 2-(4-chlorophenyl)-5-(2-furyl)-4-oxazoleacetic acid (TA-1801A) in humans through several in vitro mechanistic studies. Assessment of a panel of recombinant UGT isoforms revealed the TA-1801A glucuronosyltransferase activity of UGT1A1, UGT1A3, UGT1A7, UGT1A9, and UGT2B7. Kinetic analyses of the TA-1801A glucuronidation by recombinant UGT1A1, UGT1A3, UGT1A9, and UGT2B7 showed that the \( K_m \) value for UGT2B7 was apparently consistent with those in human liver and jejunal microsomes. The TA-1801A glucuronosyltransferase activity in human liver microsomes was inhibited by bilirubin (typical substrate for UGT1A1), propofol (typical substrate for UGT1A9), diclofenac (substrate for UGT1A9 and UGT2B7), and genistein (substrate for UGT1A1, UGT1A3, and UGT1A9). The inhibition by bilirubin, propofol, and diclofenac of the TA-1801A glucuronidation was less pronounced in jejunal microsomes than liver microsomes, suggesting that the contribution of UGT1A1, UGT1A9, and UGT2B7 to the TA-1801A glucuronidation is smaller in the intestine than the liver. In contrast, genistein strongly inhibited the TA-1801A glucuronosyltransferase activity in both human liver and jejunal microsomes. These results suggest that the glucuronidation of TA-1801A is mainly catalyzed by UGT1A1, UGT1A9, and UGT2B7 in the liver, and by UGT1A1, UGT1A3, and UGT2B7 in the intestine in humans.

Key words: glucuronidation; human UGTs; liver microsomes; intestinal microsomes; enzyme kinetics

Introduction

When TA-1801,\(^1\) an ester prodrug of TA-1801A [2-(4-chlorophenyl)-5-(2-furyl)-4-oxazoleacetic acid, Fig. 1], was administered orally to humans, the glucuronide of TA-1801A was the most abundant metabolite in human urine.\(^2\) There were significant species and tissue differences in the TA-1801A glucuronosyltransferase activities. It was demonstrated that the activity of the TA-1801A glucuronidation in human intestinal microsomes is as well as that in human liver microsomes.\(^3\)

To date, 18 functional UDP-glucuronosyltransferase (UGT) isoforms have been identified in humans and classified on the basis of primary amino acid sequence into two families of protein termed UGT1 and UGT2.\(^4\) UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, and UGT2B17 are expressed in human liver.\(^4\) UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A10, UGT2B4, UGT2B7, UGT2B10, and UGT2B15 are expressed in human intestine.\(^4\) Several genetic polymorphisms have been identified in the UGT
Identification of UGT(s) Responsible for TA-1801A

Materials and Methods

Materials: TA-1801A was synthesized at Tanabe Seiyaku Co., LTD. (Saitama, Japan). UDP-glucuronic acid, alamethicin, propofol, and genistein were purchased from Sigma-Aldrich (St. Louis, MO). Bilirubin was obtained from Wako Pure Chemicals (Osaka, Japan), and diclofenac was from Ultrafine Chemicals and Research (Manchester, UK). Pooled human liver microsomes from donors (13 males, 4–62 years old; 9 females, 40–74 years old) were purchased from BD Gentest (Woburn, MA). Recombinant human UGTs (UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in baculovirus-infected insect cells (Supersomes) were also from BD Gentest. Pooled human jejunal microsomes from donors (five males, 19–55 years old; five females, 16–51 years old) were obtained from Tissue Transformation Technologies (Edison, NJ). The protein contents were used as described in the data sheets provided by the manufacturer. All other chemicals and solvents were of the highest grade commercially available.

TA-1801A glucuronidation assay: TA-1801A glucuronosyltransferase activities in human liver and jejunal microsomes, and recombinant UGTs were determined as described previously. Briefly, a typical incubation mixture (100-μL total volume) contained 50 mM Tris-HCl buffer, pH 7.5, 8 mM MgCl₂, 2 mM UDP-glucuronic acid, 25 μg/mL alamethicin, 0.2 mg/mL human liver microsomes (human jejunal microsomes or recombinant UGTs), and 12.5, 20, or 100 μM TA-1801A. The glucuronosyltransferase activities of 12 recombinant UGTs were determined in assays using HPLC, and 7-hydroxy 4-trifluoromethylcoumarin or trifluoperazine as substrates previously. We confirmed that all recombinant UGTs were enough active to determine the TA-1801A glucuronidation.

Kinetic analyses: The kinetics studies were performed using recombinant UGTs (UGT1A1, UGT1A3, UGT1A9, and UGT2B7) expressed in microsomes. In determining the kinetic parameters, the TA-1801A concentration ranged from 1.56 to 400 μM. Kinetic parameters were estimated from the fitted curves using the Prism computer program (GraphPad Software Inc., San Diego, CA), designed for nonlinear regression analysis. The following equations were applied for Michaelis-Menten kinetics (eq. 1), substrate inhibition kinetics (eq. 2), or Hill equation (eq. 3) previously:

\[ V = V_{\text{max}} \times \frac{[S]}{K_m + [S]} \]  
\[ V = V_{\text{max}} \times \frac{[S]}{K_m + [S] + [S]^2/K_s} \]  
\[ V = V_{\text{max}} \times \frac{[S]^n}{(S_0^n + [S]^n)} \]

where \( V \) is the rate of reaction, \( V_{\text{max}} \) is the maximum velocity, \( K_m \) is Michaelis constant (substrate concentration at 0.5 \( V_{\text{max}} \)), \([S]\) is the substrate concentration, \( K_s \) is the constant describing the substrate inhibition interaction, \( S_0 \) is the substrate concentration resulting in 50% of \( V_{\text{max}} \) (analogous to \( K_m \)), and \( n \) is the Hill coefficient.

Inhibition analysis of TA-1801A glucuronosyltransferase activities in human liver and jejunal microsomes: Four compounds were tested for their inhibitory effects on the TA-1801A glucuronosyltransferase activity. Bilirubin is a typical substrate for UGT1A1. Propofol is a typical substrate for UGT1A1. Diclofenac is a substrate for UGT1A9 and UGT2B7. Genistein is a substrate for UGT1A1, UGT1A3, and UGT1A9. Bilirubin, propofol, diclofenac, and genistein were dissolved in dimethyl sulfoxide, with the solvent being used as the control. The final concentration of organic solvents (the mixture of methanol and dimethyl sulfoxide) in the reaction mixture was 2% (v/v). Organic solvents tested up to 2% (v/v) had no effect on the formation of TA-1801A glucuronide. The inhibitor concentrations for IC₂₅ and IC₅₀ determinations in human liver or jejunal microsomes were 1 to 300 μM. Each incubation contained 0.2 mg/mL microsomal protein. The glucuronosyltransferase activities in human liver and jejunal microsomes at 20 μM TA-1801A were determined as described above. The IC₂₅ and IC₅₀ values were estimated graphically.

Selectivity of UGT inhibitors towards recombinant UGTs: For the determination of the inhibitory effects of various UGT substrates on recombinant UGT1A1, UGT1A3, UGT1A9, and UGT2B7, the inhibitor concentrations were chosen at or near IC₂₅ and IC₅₀ in human liver microsomes. Each incubation contained 0.3 mg/mL microsomal protein. The glucuronosyltransferase activities of recombinant UGTs (UGT1A1,
UGT1A3, UGT1A9, and UGT2B7) expressed in microsomes at 20 µM TA-1801A were determined as described above.

**Results**

**TA-1801A glucuronidation in recombinant UGT isoforms:** All recombinant UGT isoforms expressed in baculovirus-infected insect cells were used to determine their TA-1801A glucuronosyltransferase activities (Fig. 2). At high substrate concentration (100 µM), UGT1A1, UGT1A9, and UGT2B7 exhibited high TA-1801A glucuronosyltransferase activities, in addition, UGT1A3 and UGT1A7 exhibited TA-1801A glucuronosyltransferase activities. At middle substrate concentration (12.5 µM), UGT1A1, UGT1A9, and UGT2B7 exhibited the TA-1801A glucuronosyltransferase activities. All other isoforms (UGT1A4, UGT1A6, UGT1A8, UGT1A10, UGT2B4, UGT2B15, and UGT2B17) had very low TA-1801A glucuronosyltransferase activities (<10 pmol/min/mg protein) or no activities.

**Kinetics of TA-1801A glucuronidation in recombinant UGTs:** Kinetics analyses of the TA-1801A glucuronidation in recombinant human UGT1A1, UGT1A3, UGT1A9, and UGT2B7 were performed. As shown in Fig. 3, A and D, the TA-1801A glucuronidation by UGT1A1 and UGT2B7 displayed typical Michaelis-Menten kinetics. In contrast, UGT1A3-catalyzed glucuronidation followed a sigmoidal kinetic (Fig. 3B), which manifests as a curvilinear Eadie-Hofstee plot. In addition, glucuronidation by UGT1A9 showed a complex kinetic with decreasing velocity at higher substrate concentration (Fig. 3C), suggesting the effect of substrate inhibition. Fitting the data points to the eq. 1, 2, or 3 yielded the kinetic parameters listed in Table 1. The $K_m$ values for recombinant UGT1A1 and UGT2B7 were 138±5.9 and 45.6±2.8 µM, respectively. Additionally, the $S_{50}$ value for recombinant UGT1A3 was 293±67 µM. With respect to recombinant UGT1A9, the $K_m$ value was 11.9±0.7 µM.

**Inhibition analyses of TA-1801A glucuronidation in human liver and jejunum microsomes:** The inhibitory effects of bilirubin (UGT1A1), propofol (UGT1A9), diclofenac (UGT1A9 and UGT2B7), and genistein (UGT1A1, UGT1A3, and UGT1A9) on the TA-1801A glucuronosyltransferase activities in human liver and jejunum microsomes were determined. As shown in Fig. 4A, the TA-1801A glucuronosyltransferase activity in human liver microsomes was prominently inhibited by diclofenac and genistein (IC$_{25}$ and IC$_{50}$ are 10 and 36 µM for diclofenac, 4 and 15 µM for genistein, respectively). The inhibitory effect of propofol (IC$_{25}$ and IC$_{50}$ are 31 and 111 µM, respectively) was also confirmed, and bilirubin partly inhibited the activity by approximately 40% (IC$_{25}$ and IC$_{50}$ are 2 and >300 µM, respectively). In contrast, the TA-1801A glucuronosyltransferase activity in human jejunum microsomes was prominently inhibited by diclofenac and genistein (IC$_{25}$ and IC$_{50}$ are 10 and 36 µM for diclofenac, 4 and 15 µM for genistein, respectively). The inhibitory effect of propofol (IC$_{25}$ and IC$_{50}$ are 31 and 111 µM, respectively) was also confirmed, and bilirubin partly inhibited the activity by approximately 40% (IC$_{25}$ and IC$_{50}$ are 2 and >300 µM, respectively). In contrast, the TA-1801A glucuronosyltransferase activity in human jejunum microsomes was prominently inhibited by genistein (IC$_{25}$ and IC$_{50}$ are 10 and 20 µM, respectively) as shown in Fig. 4B. The inhibitory effects of diclofenac (IC$_{25}$ and IC$_{50}$ are 21 and 138 µM, respectively), propofol (IC$_{25}$ and IC$_{50}$ are 86 and 285 µM, respectively), and bilirubin (IC$_{25}$ and IC$_{50}$ are 40 and >300 µM, respectively) were moderately.

**Selectivity of UGT inhibitors towards recombinant UGTs:** To determine the UGT isoform selectivity of inhibition by compounds, we investigated the inhibitory effects on the TA-1801A glucuronidation by recombinant human UGT isoforms (Fig. 5). Bilirubin strongly inhibited the TA-1801A glucuronosyltransferase activity of UGT1A1 by 64.7% at 3 µM, and 95.7% at 100.

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**Table 1.** The $K_m$ values for recombinant UGT1A1 and UGT2B7 were 138±5.9 and 45.6±2.8 µM, respectively. Additionally, the $S_{50}$ value for recombinant UGT1A3 was 293±67 µM. With respect to recombinant UGT1A9, the $K_m$ value was 11.9±0.7 µM.
The concentration of TA-1801A ranged from 1.56 to 400 μM. TA-1801A glucuronosyltransferase activities were determined as described under Materials and Methods. Each inset shows the Eadie-Hofstee plot of the experimental data. Each incubation was performed by triplicate determinations.

Table 1. Kinetic parameters of TA-1801A glucuronidation in recombinant UGT isoforms and in human liver (HLM) and jejunum microsomes (HJM)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_m$ or $S_{50}$ (μM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$K_{si}^{a)}$ (μM)</th>
</tr>
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<tbody>
<tr>
<td>UGT1A1b)</td>
<td>138 ± 5.9</td>
<td>0.64 ± 0.012</td>
<td>—</td>
</tr>
<tr>
<td>UGT1A3c)</td>
<td>293 ± 67</td>
<td>0.25 ± 0.033</td>
<td>—</td>
</tr>
<tr>
<td>UGT1A9d)</td>
<td>11.9 ± 0.7</td>
<td>0.32 ± 0.006</td>
<td>1560 ± 228</td>
</tr>
<tr>
<td>UGT2B7b)</td>
<td>45.6 ± 2.8</td>
<td>0.43 ± 0.008</td>
<td>—</td>
</tr>
<tr>
<td>HLMb,e)</td>
<td>64.1 ± 2.7</td>
<td>1.51 ± 0.022</td>
<td>—</td>
</tr>
<tr>
<td>HJMb,e)</td>
<td>50.4 ± 3.3</td>
<td>2.42 ± 0.050</td>
<td>—</td>
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</tbody>
</table>

The concentration of TA-1801A ranged from 1.56 to 400 μM. The kinetic parameters were calculated with GraphPad Prism software. Each value represents best-fit values ± S.E. of triplicate points. Kinetic plots are presented in Fig. 3. 

Table 1. Kinetic parameters of TA-1801A glucuronidation in recombinant UGT isoforms and in human liver (HLM) and jejunum microsomes (HJM)

- $K_m$ or $S_{50}$ = substrate inhibition constant.
- b) Michaelis-Menten.
- c) Hill equation ($n = 1.21 ± 0.10$).
- d) Substrate inhibition.
- e) Taken from a previous report.2)

μM, respectively, whereas the inhibitory effect was very weak for UGT1A3, UGT1A9, and UGT2B7. Propofol effectively inhibited the TA-1801A glucuronosyltransferase activity of UGT1A9 by 33.5% at 30 μM, and 65.4% at 100 μM, respectively. The inhibitory effect at 30 μM was weak for UGT1A1 and UGT2B7, but at 100 μM was moderate for them, whereas was very weak for UGT1A3. Diclofenac effectively inhibited the TA-1801A glucuronosyltransferase activity of UGT2B7 by 40.6% at 10 μM, and 67.0% at 30 μM, respectively. The inhibitory effect was relatively weak for UGT1A1 and UGT1A9, whereas was very weak for UGT1A3. On the other hand, genistein inhibited the TA-1801A glucuronosyltransferase activities of UGT1A1 (by 38.8% at 3 μM, and 97.6% at 30 μM, respectively), UGT1A3 (by 11.4% at 3 μM, and 47.4% at 30 μM, respectively), and UGT1A9 (by 25.4% at 3 μM, and 79.5% at 30 μM, respectively). The inhibitory effect was relatively weak for UGT2B7.

Discussion

In the present study, the metabolism of TA-1801A to its glucuronide was investigated with several insect cell microsomes expressing human UGTs as well as human liver and jejunum microsomes. Consequently, recombinant UGT1A1, UGT1A9, and UGT2B7 showed significant glucuronosyltransferase activities both at middle and high substrate concentrations among the UGT isoforms examined (Fig. 2). Recombinant UGT1A3 and
Fig. 4. Inhibitory effects of typical substrates for UGT isoforms on TA-1801A glucuronosyltransferase activity in human liver (A) or jejunum (B) microsomes.

TA-1801A glucuronosyltransferase activities at 20 μM TA-1801A in pooled human liver and jejunum microsomes were determined as described under Materials and Methods. Bilirubin (UGT1A1), propofol (UGT1A9), diclofenac (UGT1A9 and UGT2B7), and genistein (UGT1A1, UGT1A3, and UGT1A9) were used as inhibitors. Control activities in the pooled human liver and jejunum microsomes in the absence of inhibitor were 348 ± 10.4 and 660 ± 18.0 pmol/min/mg protein, respectively. Each value represents the mean ± S.D. of triplicate determinations.

Fig. 5. Selectivity of UGT inhibitors towards recombinant UGT1A1, UGT1A3, UGT1A9, and UGT2B7.

Each incubation contained 0.3 mg/mL of microsomal protein, 2 mM UDP-glucuronic acid, 25 μg/mL of alamethicin and 20 μM TA-1801A. Control activities (pmol/min/mg protein) were: UGT1A1, 98 ± 5.9; UGT1A3, 10 ± 0.7; UGT1A9, 210 ± 3.4; and UGT2B7, 159 ± 3.1. Each concentration (μM) of the inhibitors was: bilirubin, 3 and 100; propofol, 30 and 100; diclofenac, 10 and 30; genistein, 3 and 30. Each column represents the mean ± S.D. of triplicate determinations.

UGT1A7 also catalyzed the TA-1801A glucuronidation, although it has been reported that UGT1A7 is expressed in human esophagus and stomach, but not in human liver and intestine.4,21) These findings suggest that multiple UGT isoforms are involved in the glucuronidation of TA-1801A in human liver and intestine. Furthermore, to clarify the contribution among UGT enzymes to the TA-1801A glucuronidation we performed kinetic analyses of recombinant human UGT microsomes, as was the case with human liver and jejunum microsomes.
In the previous study, the kinetic pattern of the TA-1801A glucuronidation was monophasic in human liver and jejunum microsomes.² The $K_m$ value for recombinant UGT2B7 of the TA-1801A glucuronidation was approximately 20-fold less than the levels of UGT1A1,²¹ it was reported that hepatic UGT1A3 mRNA levels were apparently consistent with those in human liver and jejunum microsomes (Table 1). However, these results would not contradict the involvement of other UGTs (such as UGT1A1 and UGT1A9) on the TA-1801A glucuronidation in human liver and intestine, because the $K_m$ values for UGT1A1 and UGT1A9 are less different (within 4-fold) from that for UGT2B7.

Next, to confirm the involvement of multiple UGT isoforms on the TA-1801A glucuronidation in human liver and intestine, we performed the inhibition analyses using typical substrates for UGT isoforms. Bilirubin is well known to be a specific substrate for UGT1A1.¹⁴,¹⁵ Under this condition in the present study, bilirubin selectively inhibited the TA-1801A glucuronyltransferase activity of recombinant UGT1A1, but not UGT1A3, UGT1A9, and UGT2B7 (Fig. 5). At a concentration of 100 μM, bilirubin almost completely inhibited the TA-1801A glucuronosyltransferase activity of recombinant UGT1A1, whereas those in human liver and jejunum microsomes were inhibited by approximately 40% (Fig. 4). These results suggest that the contribution of UGT1A1 to the TA-1801A glucuronidation in human liver and intestine is no less than 40% of all. Propofol is known to be a typical substrate for UGT1A9.¹⁶,¹⁷ Additionally, diclofenac is known to be a substrate for UGT1A9 and UGT2B7.¹⁸,¹⁹ In our experiments, propofol inhibited the TA-1801A glucuronosyltransferase activity of UGT2B7, whereas the inhibitory effect was relatively weak for UGT1A1, UGT1A3, and UGT1A9 (Fig. 5). Diclofenac and, to a lesser extent, propofol inhibited the TA-1801A glucuronosyltransferase activity in human liver microsomes. In addition, genistein, known to be a substrate for UGT1A1, UGT1A3, and UGT1A9,²⁰ strongly inhibited the TA-1801A glucuronosyltransferase activity in human liver microsomes (Fig. 4A). Since it has been reported that hepatic UGT1A3 mRNA levels were approximately 20-fold less than the levels of UGT1A1,²¹ it is unlikely that UGT1A3 would play a role in the glucuronidation of TA-1801A in human liver. The inhibitory effects observed with these inhibitors in human liver microsomes suggest that the glucuronidation of TA-1801A is mainly catalyzed by UGT1A1, UGT1A9, and UGT2B7 in human liver.

Data in Fig. 4 indicate that the inhibition by bilirubin, propofol, and diclofenac of the TA-1801A glucuronidation was less pronounced in jejunal microsomes than liver microsomes, suggesting that the contribution of UGT1A1, UGT1A9, and UGT2B7 to the TA-1801A glucuronidation is smaller in the intestine than the liver. In contrast, genistein strongly inhibited the TA-1801A glucuronosyltransferase activity in both human liver and jejunal microsomes. It is considered that this phenomenon would be due to the contribution of UGT1A3 to the TA-1801A glucuronidation. It has been reported that UGT1A9 is expressed in human liver, but not in human intestine,⁴,²² whereas Albert et al.²³ reported that UGT1A9 was also expressed in human intestine. From these reports and the inhibition effects of propofol in the present study, it might be mentioned that the contribution of UGT1A9 to the TA-1801A glucuronidation was small in human jejunal microsomal samples used. Therefore, taking into account the inhibition observed with these inhibitors in human jejunal microsomes, it is suggested that the glucuronidation of TA-1801A is mainly catalyzed by UGT1A1, UGT1A3, and UGT2B7 in human intestine. It has been reported that the majority of carboxylic acid compounds are glucuronidated principally by UGT1A3, UGT1A9, and UGT2B7.⁴,¹¹,²⁴ Bilirubin, which is also metabolized to an acyl glucuronide, is selectively glucuronidated by UGT1A1. However, bilirubin glucuronidation by UGT1A1 is a rare example of the ability of this enzyme to form carboxylic ester glucuronides, because other carboxylic acids are not glucuronidated by this protein.¹⁵,²⁴ The present results were similar to these reports. But, it is interesting that UGT1A1 catalyzes the metabolism of the carboxylic acid such as TA-1801A.

In conclusion, we characterized the hepatic and intestinal UGT isoforms responsible for the glucuronidation of TA-1801A in humans through the activities and kinetics of recombinant human UGTs, and the inhibition analyses using typical substrates for UGT isoforms. These results suggest that the glucuronidation of TA-1801A is mainly catalyzed by UGT1A1, UGT1A9, and UGT2B7 in the liver, and by UGT1A1, UGT1A3, and UGT2B7 in the intestine in humans.

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References


