Effects of Ketoconazole on Glucuronidation by UDP-Glucuronosyltransferase Enzymes
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Abstract Purpose: Ketoconazole has been shown to inhibit the glucuronidation of the UGT2B7 substrates zidovudine and lorazepam. Its effect on UGT1A substrates is unclear. A recent study found that coadministration of irinotecan and ketoconazole led to a significant increase in the formation of SN-38 (7-ethyl-10-hydroxycamptothecine), an UGT1A substrate. This study investigates whether ketoconazole contributes to the increase in SN-38 formation by inhibiting SN-38 glucuronidation.

Experimental Design: SN-38 glucuronidation activities were determined by measuring the rate of SN-38 glucuronide (SN-38G) formation using pooled human liver microsomes and cDNA-expressed UGT1A isoforms (1A1, 1A7 and 1A9) in the presence of ketoconazole. Indinavir, a known UGT1A1 inhibitor, was used as a positive control. SN-38G formation was measured by high-performance liquid chromatograph.

Results: Ketoconazole competitively inhibited SN-38 glucuronidation. Among the UGT1A isoforms screened, ketoconazole showed the highest inhibitory effect on UGT1A1 and UGT1A9. The Ki values were 3.3 ± 0.8 μmol/L for UGT1A1 and 31.9 ± 3.3 μmol/L for UGT1A9.

Conclusions: These results show that ketoconazole is a potent UGT1A1 inhibitor, which seems the basis for increased exposure to SN-38 when coadministered with irinotecan.

Irinotecan was approved for the first-line treatment of metastatic colorectal cancer in combination with 5-flurouracil and leucovorin following the positive results of two large randomized control trials showing improvement in treatment survival (1, 2). Irinotecan has a relatively complex metabolic pathway. It is converted to its active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), predominantly by carboxylesterase 2 in the liver (3, 4). Irinotecan undergoes oxidative biotransformation by CYP3A4 and CYP3A5 to several inactive metabolites (5, 6). SN-38, in turn, is detoxified primarily by UGT1A1 to SN-38 glucuronide (SN-38G; refs. 7–10). UGT1A9 may also have a minor role in the hepatic clearance of SN-38, whereas UGT1A7 may be important in the extrahepatic formation of SN-38G (9, 10). Functional polymorphisms in UGT1A1 have been associated with reduced catalytic efficiency and patients with UGT1A1*28 and −3156G>A variants are more likely to experience treatment related diarrhea or leucopenia when treated with irinotecan (11, 12).

Ketoconazole is a widely used antifungal agent well known for its inhibitory effect on CYP3A4 and the ABCB1 transporter (13, 14). A recent study by Kehler et al. showed that coadministration of irinotecan and ketoconazole led to a significant increase in the relative formation of SN-38 (15). Such increase was attributed to a reduction in irinotecan clearance secondary to the inhibitory effect of ketoconazole on CYP3A enzymes, although a reduction of irinotecan clearance was not observed in the study. An alternative hypothesis would be that ketoconazole inhibited SN-38 glucuronidation resulting in an increase in SN-38 AUC.

Ketoconazole has also been shown to inhibit the glucuronidation of UGT2B7 substrates zidovudine and lorazepam in in vitro studies (16–18). A recent study by Satoh et al. also showed that ketoconazole inhibited UGT1A1-mediated glucuronidation of estradiol using human liver microsomes (19). In this study, we investigated the inhibitory effects of ketoconazole on SN-38 glucuronidation in vitro using pooled human liver microsomes, cDNA-expressed isoforms of UGT1A, and human hepatocytes.

Materials and Methods

Chemicals and reagents. Irinotecan and SN-38 were kindly provided by Dr. Kiyoshi Terada (Yakult Honsha Co., Ltd., Japan). Indinavir was obtained from Merck & Co., Inc. (Whitehouse Station, NJ). Fluconazole was obtained from Pfizer (New York, NY). Radiolabeled uridine diphosphoglucuronic acid (14C-UDPGLA) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Ketoconazole, cyclosporine A, bilirubin, L-α-phosphatidylcholine, saccharolactone (D-saccharic acid 1,4-lactone), camptothecin, dihydrocarbamazepine, and UDPGA were all

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obtained from Sigma-Aldrich (St. Louis, MO). All other reagents and high-performance liquid chromatograph solvents were of the highest quality commercially available.

Human hepatocytes in a 12-well collagen culture plate, from a single donor, were purchased from In Vitro Technologies (Baltimore, MD). Human recombinant uridine diphosphate glucuronosyltransferases (UGT1A1, UGT1A7, and UGT1A9) produced using baculovirus (Autographa california)–transfected insect cells (BTI-TN-5B1-4) were purchased from Gentest (Woburn, MA).

Human liver microsome preparation. Human liver samples from 10 separate donors were obtained from the Liver Tissue Procurement and Distribution System (NIH N01-DK-9-2310, Pittsburgh, PA). Microsomes were prepared by differential centrifugation methods (20). Total protein content in microsomes was determined by the Bradford method using bovine serum albumin as the standard (21).

In vitro studies with microsomes and cDNA-expressed UGT1A isoforms. The IC_{50} values for inhibition of SN-38 glucuronidation by ketoconazole and indinavir, a known UGT1A1 inhibitor (22), were determined using human liver microsomes. The concentrations of ketoconazole and indinavir ranged from 100 nmol/L to 500 nmol/L and from 100 nmol/L to 1 mmol/L, respectively. The incubation mixture (200 μL) contained SN-38 (5 μmol/L), MgCl_{2} (10 mmol/L), Tris-HCl buffer at pH 7.4 (25 mmol/L), and microsomal protein (1 mg/mL) with appropriate inhibitors. After preincubation at 37°C for 10 minutes, the reaction was started with the addition of UDPGA (5 mmol/L). Reaction mixtures were incubated for 30 minutes, after which 400 μL of cold methanol were added to stop the reaction. Fifty microliters of 2 N/C2 HCl were added to each sample to hydrolyze the glucuronides from unreacted UDPGA (26). Ketoconazole (10 μmol/L) was added to examine its effect on bilirubin glucuronidation. Briefly, human liver microsomes (1 mg/mL) were incubated with bilirubin (340 μmol/L), 1-α-phosphatidylcholine (0.75 mg/mL), saccharolactone (8.5 mmol/mL), MgCl_{2} (10 mmol/L), and 14C-UDPGA (0.05 μCi) in a 0.5 mol/L Tris-HCl buffer (pH 7.4) for 60 minutes at 37°C. The reaction was stopped with 100 μL of cold 1% Triton X-100 in 0.7 mol/L glycine/HCl buffer (pH 2.2). Radiolabeled bilirubin glucuronides were extracted into 1 mL ethyl acetate. Three hundred–microliter aliquots were measured by liquid scintillation counting (Tri-Carb 4530; Packard, Meriden, CT).

Data analysis. Results were presented as mean values ± SE. All experiments were carried out in duplicate, except for the bilirubin assays, which were done in triplicate. Appropriate enzyme kinetic models were fitted to enzyme kinetic data by nonlinear regression analysis using GraphPad software version 4.01 (GraphPad Software, Inc., San Diego, CA) to derive K_{m} and V_{max}. Both the Michaelis-Menten (Eq. A) and the substrate activation models (Eq. B) were used, where [A] is defined as the concentration of substrate A and n is the number of binding sites:

\[
v = \frac{[A] \times V_{\text{max}}}{([A] + K_{m})}
\]

(A)

\[
v = \frac{[A] n \times V_{\text{max}}}{([A] n + K_{m} n)}
\]

(B)

The best fit was based on a number of criteria which included visual inspection of the data plots, the distribution of the residuals, the size of the sum of squared residuals, the SE of the estimates, and the F ratio test. The type of inhibition was evaluated using graphical analysis with Lineweaver-Burke plots. Initial K_{i} values were estimated using Dixon plots. A more accurate value of K_{i} was determined by fitting the kinetic data into a competitive inhibition model (Eq. C) using nonlinear regression analysis:

\[
v = \frac{[A] \times V_{\text{max}}}{([A] + K_{m} / (1 + 1/K_{i}))}
\]

(C)

Results

Effects of ketoconazole, indinavir, cyclosporine A, and fluconazole on SN-38G formation in pooled human liver microsomes. Ketoconazole and indinavir both inhibited SN-38 glucuronidation as described below. The incubation time selected was based on our previous optimization study with human liver microsomes.

High-performance liquid chromatograph analysis of SN-38G formation. The SN-38 glucuronidation assay was adapted from a previously described method (8). Twenty-microliter aliquots were injected into a high-performance liquid chromatograph assay system coupled to a fluorescence detector (Hitachi Instruments, Inc., San Jose, CA). A µBondapak C18 column (3.9 × 300 mm, 10 μm, 125 Å, Waters Corp., Milford, MA) with a Novapak guard column (4 μm, 60 Å, Waters) was used with fluorescence detection using an excitation frequency of 355 nm and an emission frequency of 515 nm. The mobile phase A consisted of 8:4:88 acetonitrile/tetrahydrofuran/0.9 mmol/L 1-heptanesulfonic acid in 50 mmol/L potassium phosphate buffer (pH 4.0) and mobile phase B was a mix of 35:65 acetonitrile/5 mmol/L 1-heptanesulfonic acid in 50 mmol/L potassium phosphate buffer (pH 4.0). Elution was done at the flow rate of 0.9 mL/min using the following gradient: 0 to 7 minutes, 100% A; 7 to 25 minutes, 100% B; and 25 to 35 minutes, 100% A. Irinotecan was used as the internal standard in studies involving human liver microsomes and recombinant UGTs, whereas camptothecin was used in the experiments with hepatocytes. The retention times for SN-38G, irinotecan, camptothecin, and SN-38 were 13.3, 15.7, 17.1, and 17.8 minutes, respectively.

Bilirubin glucuronidation assay. The bilirubin glucuronidation assay was based on a previously described method with slight modification using 14C-UDPGA and an ethyl acetate extraction to separate glucuronides from unreacted UDPGA (26). Ketoconazole (10 μmol/L) was added to examine its effect on bilirubin glucuronidation. Briefly, human liver microsomes (1 mg/mL) were incubated with bilirubin (340 μmol/L), 1-α-phosphatidylcholine (0.75 mg/mL), saccharolactone (8.5 mmol/mL), MgCl_{2} (10 mmol/L), and 14C-UDPGA (0.05 μCi) in a 0.5 mol/L Tris-HCl buffer (pH 7.4) for 60 minutes at 37°C. The reaction was stopped with 100 μL of cold 1% Triton X-100 in 0.7 mol/L glycine/HCl buffer (pH 2.2). Radiolabeled bilirubin glucuronides were extracted into 1 mL ethyl acetate. Three hundred–microliter aliquots were measured by liquid scintillation counting (Tri-Carb 4530; Packard, Meriden, CT).
glucuronidation, with IC₅₀ values of 11 and 94 μmol/L, respectively (Fig. 1). Cyclosporine A and fluconazole showed minimal inhibitory effect (3.2% and 6.3%, respectively) on the formation of SN-38G by human liver microsomes.

Effects of ketoconazole, indinavir, cyclosporine A, and fluconazole on SN-38G formation in cDNA-expressed isoforms. The effects of ketoconazole and indinavir on SN-38 glucuronidation were tested on cDNA-expressed UGT1A1, UGT1A7, and UGT1A9 (Fig. 2). Ketoconazole showed the highest inhibitory effect on UGT1A1 and UGT1A9. At the concentration of 10 μmol/L, it reduced SN-38 glucuronidation of both UGT isoforms by ∼35%. Indinavir, at 10 μmol/L, inhibited all the UGT1A isoforms tested showing the highest inhibitory effect on UGT1A9.

As ketoconazole exhibited no inhibitory effect on UGT1A7 within the range of concentrations studied (up to 500 μmol/L), only UGT1A1 and UGT1A9 were selected for further kinetic studies. The kinetics of SN-38 glucuronidation by UGT1A9 fit a Michaelis-Menten model, whereas UGT1A1 exhibits a substrate activation model. The $K_m$, $V_{max}$, and $V_{max}/K_m$ for UGT1A1 were 33.5 ± 2.8 μmol/L, 204.3 ± 7.4 pmol/min/mg protein, and 6.1 μL/min/mg protein, and for UGT1A9 were 39.6 ± 1.3 μmol/L, 29.6 ± 0.3 pmol/min/mg protein, and 0.75 μL/min/mg protein, respectively. A Lineweaver-Burke plot was used to evaluate the mechanism of inhibition of SN-38 glucuronidation by ketoconazole. Ketoconazole competitively inhibited SN-38 glucuronidation by UGT1A1 and UGT1A9 (Fig. 3). The $K_i$ values were estimated to be 3.3 ± 1.1 μmol/L for UGT1A1 and 31.9 ± 4.7 μmol/L for UGT1A9. Representative Dixon plots for ketoconazole inhibition of SN-38 glucuronidation by UGT1A1 and UGT1A9 are shown in Fig. 4.

Cyclosporine A and fluconazole were screened for their effect on SN-38 glucuronidation by UGT1A1, UGT1A7, and UGT1A9. At 1 μmol/L, cyclosporine A showed a small (<20%) but demonstrable inhibitory effect on UGT1A7 and UGT1A9 activities (18% and 14%, respectively). Fluconazole, on the other hand, did not inhibit the UGT isoforms tested (data not shown).

In vitro glucuronidation of bilirubin by pooled human liver microsomes in the presence of ketoconazole and indinavir. To confirm ketoconazole’s inhibitory effect on UGT1A1, bilirubin glucuronidation was studied in human using human liver microsomes. Ketoconazole and indinavir inhibited the glucuronidation of bilirubin, with IC₅₀ values of 53 and 69 μmol/L, respectively.

Effects of ketoconazole on irinotecan metabolism in human hepatocytes. The aim of this experiment was to assess effects of ketoconazole on the disposition of irinotecan in human hepatocytes, a system with intact UGTs, CYP3A, and transporters. Table 1 shows the formation of SN-38 and SN-38G at
two different substrate concentrations. A reduction of SN-38G concentration in the medium was observed when ketoconazole was added to the reaction at both low and high substrate concentrations. At the lower substrate concentration tested (10 μmol/L irinotecan), the formation of SN-38G was reduced by 39% and 81%, with ketoconazole at 1.6 and 16 μmol/L, respectively. At high substrate concentration (100 μmol/L irinotecan), a reduction in SN-38G formation by 44% and 83% was seen in the presence of ketoconazole at 1.6 and 16 μmol/L, respectively. When normalized for SN-38 formation, the relative formation of SN-38G (SN-38G/SN-38 ratio) remained reduced. A similar picture was observed in cell lysates, with a reduction of SN-38G formation in the presence of ketoconazole (data not shown). A modest increase in SN-38 formation (39%) was observed only when ketoconazole (1.6 μmol/L) was incubated at the lower irinotecan concentration tested (10 μmol/L).

Discussion

We report that ketoconazole exhibits a significant inhibitory effect on SN-38 glucuronidation by UGT1A1 and UGT1A9 and our findings are consistent with those of Satoh et al. (19). Inhibition of UGTs could lead to clinically significant interactions for drugs with a narrow therapeutic index (27–30). Glucuronidation is an important conjugation pathway, which accounts for the majority of phase II biotransformations. It prevents the accumulation of potential toxic compounds by converting lipophilic substrates to more soluble hydrophilic glucuronides, allowing for excretion via urine or bile (31). The UGTs have a broad spectrum of substrates including endogenous compounds (e.g., thyroid hormones, hydroxyl estradiol, and bilirubin), drugs (e.g., epirubicin, propofol, opiates, and zidovudine), environmental pollutants, and carcinogenic chemicals (32, 33).

Our kinetic analysis suggests that ketoconazole inhibited the formation of SN-38G via competitive inhibition. To investigate if ketoconazole is a substrate for UGT1A1, we incubated ketoconazole with 14C-UDPGA in pooled human liver microsomes and did not detect the presence of radiolabeled glucuronide of ketoconazole (data not shown). Although glucuronidation is a minor elimination pathway for anotherazole, fluconazole (34), ketoconazole is not known to be a substrate for UGT enzymes. Recent studies have suggested...
The formation of SN-38 by carboxylesterase 2 and elimination of SN-38G are predominantly mediated by UGT1A1 isoform. ketoconazole is a potent inhibitor of UGT1A1 and may function as a substrate transporter for UGTs and inhibit UGT1A1, thereby offsetting the effect of CYP3A4 on SN-38 formation. Our findings support the conclusion that the change in relative SN-38 formation was caused by ketoconazole. In addition, microsomes that were incubated with irinotecan and ketoconazole, we did not see an increase in the formation of SN-38 (data not shown) lending further support that the change in relative SN-38 formation was caused by inhibition of SN38 glucuronidation.

Table 1. Effects of ketoconazole on the formation of SN-38 and SN-38G in human hepatocytes

<table>
<thead>
<tr>
<th>SN-38/IS (×10⁻³)</th>
<th>Control</th>
<th>Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6 µmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 µmol/L</td>
<td></td>
<td></td>
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<tr>
<td>Irinotecan (10 µmol/L)</td>
<td>9.7 ± 0.1</td>
<td>13.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>26.9 ± 0.3</td>
<td>16.4 ± 1.4</td>
</tr>
<tr>
<td>Irinotecan (100 µmol/L)</td>
<td>64.4 ± 4.8</td>
<td>70.0 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>85.2 ± 7.2</td>
<td>46.9 ± 3.2</td>
</tr>
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</table>

NOTE: Data are presented as mean ± SE (n = 2). Abbreviation: IS, internal standard.

possible interactions between CYP enzymes and UGTs (35–37). Taura et al. showed that CYP1A1 may function as a substrate transporter for UGTs and inhibition of CYP1A1 could interfere with glucuronidation activity. However, we have shown, by using cDNA-expressed UGT1As, that ketoconazole can inhibit SN-38 glucuronidation in the absence of CYP enzymes.

In human hepatocytes, we observed that SN-38G formation was reduced by ketoconazole. Increased formation of SN-38, however, was not observed at a higher ketoconazole concentration. Given that ketoconazole is a potent inhibitor of CYP3A4, UGT1A1, and UGT1A9, this observation was somewhat surprising and may indicate additional inhibitory effects of ketoconazole on irinotecan or SN-38 transport. We did not find a difference in the SN-38 and SN-38G concentration between reaction medium and cell lysates. Hence, the observation cannot be explained by the inhibition of efflux transporters of SN-38 at a higher ketoconazole dose. ketoconazole is known to inhibit influx transporters conveying the organic anion-transporting polypeptides (38). At higher concentration, ketoconazole may have inhibited the influx of irinotecan, thereby offsetting the effect of CYP3A4 and UGT1A1 inhibition. Recently, OATP1B1 (SLCO1B1), OATP2B1 (SLCO2B1), and OATP1B3 (SLCO1B3) were evaluated for their transport activity for irinotecan and its metabolites (39). OATP1B1 was found to transport SN-38 but not irinotecan and SN-38G. At this point in time, influx transporters that are involved in irinotecan have not been identified.

Kehrer et al. reported that modulation by ketoconazole resulted in 87% reduction in the relative formation of 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin, an oxidative metabolite of irinotecan, and an increase in the relative exposure to SN-38 (15). Similar to ketoconazole, coadministration of cyclosporine A with irinotecan resulted in an overall increase in irinotecan and SN-38 AUC (40–42). However, in contrast to ketoconazole, no change in the relative formation of SN-38 (ratio of SN-38 AUC to irinotecan AUC) was observed suggesting that the overall increase in SN-38 is the result of the decreased irinotecan clearance from CYP3A4 inhibition. The change in relative formation of SN-38 depends on two critical processes: the formation of SN-38 by carboxylesterase 2 and elimination of SN-38 via SN-38 glucuronidation and biliary excretion. Our finding supports the conclusion that the change in relative formation of SN-38 was primarily due to UGT1A1 inhibition by ketoconazole. In addition, when microsomes were incubated with irinotecan and ketoconazole, we did not see an increase in the formation of SN-38 (data not shown) lending further support that the change in relative SN-38 formation was caused by inhibition of SN38 glucuronidation.

With inhibition of SN-38 glucuronidation, one should expect a decrease in the relative formation of SN-38G (ratio of SN-38G AUC to SN-38 AUC). But in Kehrer et al.’s study, the relative formation of SN-38G was unchanged. It is unclear as to what could have accounted for the discrepancy. The relative formation of SN-38G may not be a good estimate of SN-38 glucuronidation activity, because it may be affected by a change in the biliary excretion of SN-38G. The clinical observation may be a reflection of the complex interaction among transporters, UGT, and CYP enzymes influenced by the relative concentration of ketoconazole and irinotecan. Additional, in vivo studies on transporters may shed light to drug-drug interaction involving ketoconazole.

In conclusion, we have shown that commonly known CYP inhibitors like ketoconazole and cyclosporine A can inhibit different UGT1A isoforms and may in part contribute to the disposition of SN-38, although the relative contribution compared with CYP3A4 and transporters inhibition is not clear. CYP3A4 and ABCB1 have been previously shown to have overlapping substrate specificity. In addition, substrates of CYP3A4 or ABCB1 frequently undergo phase II conjugation by UGT1A as well, including drugs commonly used by cancer patients (e.g., irinotecan, etoposide, and induction; refs. 43, 44). Previously reported drug-drug interactions involving ketoconazole may warrant reinterpretation in light of our findings, which may reflect a component of its effect on glucuronidation, as well as oxidation and transport. Given the widespread use of ketoconazole in cancer patients as antifungal and for treatment of prostate cancer, our study provides a strong rationale for additional clinical studies especially for drugs that are predominantly metabolized by UGT1A1 and/or UGT1A9.

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References


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