IN VITRO INHIBITION OF UDP GLUCURONOSYLTRANSFERASES BY ATAZANAVIR AND OTHER HIV PROTEASE INHIBITORS AND THE RELATIONSHIP OF THIS PROPERTY TO IN VIVO BILIRUBIN GLUCURONIDATION

Donglu Zhang, Theodore J. Chando, Donald W. Everett, Christopher J. Patten, Shangara S. Dehal, and W. Griffith Humphreys


ABSTRACT:

Several human immunodeficiency virus (HIV) protease inhibitors, including atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir, were tested for their potential to inhibit uridine 5′-diphospho-glucuronosyltransferase (UGT) activity. Experiments were performed with human cDNA-expressed enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A9, and 2B7) as well as human liver microsomes. All of the protease inhibitors tested were inhibitors of UGT1A1, UGT1A3, and UGT1A4 with IC₅₀ values that ranged from 2 to 87 μM. The IC₅₀ values found for all compounds for UGT1A6, 1A9, and 2B7 were >100 μM. The inhibition (IC₅₀) of UGT1A1 was similar when tested against the human cDNA-expressed enzyme or human liver microsomes for atazanavir, indinavir, and saquinavir (2.4, 87, and 7.3 μM versus 2.5, 68, and 5.0 μM, respectively). By analysis of the double-reciprocal plots of bilirubin glucuronidation activities at different bilirubin concentrations in the presence of fixed concentrations of inhibitors, the UGT1A1 inhibition by atazanavir and indinavir was demonstrated to follow a linear mixed-type inhibition mechanism (Kᵢ = 1.9 and 47.9 μM, respectively). These results suggest that a direct inhibition of UGT1A1-mediated bilirubin glucuronidation may provide a mechanism for the reversible hyperbilirubinemia associated with administration of atazanavir as well as indinavir. In vitro-in vivo scaling with J/P/Kᵢ predicts that atazanavir and indinavir are more likely to induce hyperbilirubinemia than other HIV protease inhibitors studied when a free Cₘₐₓ drug concentration was used. Our current study provides a unique example of in vitro-in vivo correlation for an endogenous UGT-mediated metabolic pathway.

The HIV protease is an essential enzyme that cuts the viral gag-pol polyprotein into its functional subunits. Atazanavir, indinavir, saquinavir, lopinavir, ritonavir, and nelfinavir are HIV protease inhibitors. The structures of these HIV protease inhibitors are shown in Fig. 1. As opposed to atazanavir, which is an azapeptide protease inhibitor (Goldsmith and Perry, 2003), other listed HIV protease inhibitors are peptidomimetics sharing the same structural determinant, i.e., a hydroxyethylene or a hydroxyethylamine moiety, which makes them nonscissile HIV protease substrate analogs. These HIV protease inhibitors are metabolized primarily by hepatic CYP3A enzymes, and they are also inhibitors of CYP3A enzymes (Flexner, 2000; de Maat et al., 2003; Goldsmith and Perry, 2003; Ernest et al., 2005). All of these HIV protease inhibitors have a high protein binding (>98%), except indinavir and atazanavir, which have a protein binding of 60 and 86%, respectively. Most of the HIV protease inhibitors are bound to α₁-acid glycoprotein instead of albumin (de Maat et al., 2003). There are no literature reports indicating that HIV protease inhibitors are good substrates for human UGT enzymes, although there is evidence to support indinavir as a substrate of UGTs (Balani et al., 1996).

Glucuronidation represents a major pathway for the elimination of a vast number of endogenous chemicals and xenobiotics. UGT1A1 is the only known human UGT to glucuronidate bilirubin (Burchell et al., 1995). In addition to bilirubin glucuronidation, a critical physiological metabolic reaction, UGT1A1 also catalyzes glucuronidation of carboxylic acid-containing drugs such as fluoroquinolones moxifloxacin and sitafloxacin, as well as nonsteroidal anti-inflammatory drugs sulindac, sulindac sulfone, and indomethacin (Kuehl et al., 2005; Tachibana et al., 2005). UGT2B7 is considered a very important enzyme for the glucuronidation of many drugs (Williams et al., 2004).

Bilirubin is a waste product (approximately 4 mg/kg/day produced by normal subjects) mainly derived from the degradation of hemoglobin from senescent red blood cells (Berk et al., 1974, 1979). Unconjugated bilirubin is highly bound to albumin and can lead to toxicities if the bilirubin/albumin molar ratio exceeds 1:1. Several steps are involved for elimination of bilirubin. Unconjugated bilirubin in the circulation is likely transported to the liver by the liver-specific organic anion uptake transporting polypeptide OATP1B1 (Briz et al., 2003). UGT1A1 catalyzes glucuronidation of bilirubin at one or both of the two-propionic acid groups with glucuronic acid, which is
required for the following elimination via the biliary transporter MRP2 (Kamisako et al., 2000). In humans, the glucuronidation of bilirubin is carried out exclusively by UGT1A1 with an apparent $K_m$ value of $<$10 $\mu$M, which is a lower value than most of other glucuronidation reactions (Williams et al., 2004; Luukkanen et al., 2005). The glucuronidated bilirubin may also be carried back to sinusoidal blood by MRP3 if biliary excretion pathways (e.g., MRP2) are impaired (Keppler and König, 2000). There are a number of mutations in the UGT1A1 gene that have been described that lead to decreased UGT1A1 activities and increased levels of bilirubin, i.e., patients with Crigler-Nijjar and Gilbert syndromes (Zucker et al., 2001; Miners et al., 2002). Crigler-Nijjar and Gilbert syndromes result from genetic polymorphisms in the UGT1A1 coding region and/or promoter leading to an approximately 90% and 40% reduction in bilirubin clearance, respectively (Tukey and Strassburg, 2000). Crigler-Nijjar syndrome is rare, but approximately 10% of the U.S. population has the Gilbert polymorphism. Dubin-Johnson syndrome results from the polymorphism in MRP2 transporter that associates with conjugated hyperbilirubinemia (Tukey and Strassburg, 2000).

Inhibition of bilirubin glucuronidation enzyme UGT1A1 would have potential to produce elevated unconjugated bilirubin concentration in the circulation (Brierley and Burchell, 1993). Unconjugated hyperbilirubinemia after administration of indinavir was attributed to direct inhibition of UGT1A1 by this drug (Zucker et al., 2001). Unconjugated hyperbilirubinemia has also been observed as an adverse event of atazanavir administration, however, this measurable increase is not clinically significant, and the therapy stop was not needed since the concentrations of unconjugated bilirubin never reached to a toxic level (Goldsmith and Perry, 2003). The unconjugated hyperbilirubinemia associated with atazanavir administration was rapidly reversible, suggesting that the serum bilirubin increase is a specific metabolic phenomenon and not a reflection of cellular injury (Goldsmith and Perry, 2003). To investigate whether there is a common mechanism for the unconjugated hyperbilirubinemia associated with administration of indinavir or atazanavir and to compare with other HIV protease inhibitors, atazanavir along with several other HIV protease inhibitors were tested as inhibitors of UGT1A1 and several other UGT enzymes.

Materials and Methods

Materials. Atazanavir (BMS-232632) was prepared at Bristol-Myers Squibb (Princeton, NJ). Other test substances, including indinavir, saquinavir, lopinavir, ritonavir, and nelfinavir, were commercially available. A stock solution of the test substance was prepared in 100% methanol at a concentration of 10 or 30 mM on the day of the experiment. Additional dilutions were made in methanol for each of the final inhibitor concentrations. The solvent concentrations were constant (1% final) for all incubations with and without the test substance. Human UGT Supersomes (membranes of insect cells transfected with baculovirus containing human cDNA of UGTs or microsomes of lymphoblast cells heterologously expressing human cDNA of UGT1A1) and human liver microsomes (pooled from 20 subjects) were from BD Gentest (Woburn, MA). Bilirubin, $\beta$-estradiol, 17$\beta$-estradiol 3-glucuronide, trifluoper-
dependent experiments, the bilirubin concentrations were 2, 5, 10, 20, 30, 40, and the reactions were terminated after 40 min. For substrate concentration-
g(10,000 g), the supernatant was injected onto HPLC.

Incubation Conditions with Other UGT Enzymes. Incubations were performed in triplicate for

Incubation Conditions for UGT1A1. Incubations were conducted at 37°C in a final volume of 0.2 ml in 50 mM sodium citrate buffer (pH 7.5) with 2 mM UDPGA, 25 µg/ml alamethicin, 10 mM MgCl2, and bilirubin. Bilirubin was dissolved in 100% dimethyl sulfoxide and added to the incubation to the desired final bilirubin concentration. The final microsomal protein concentration was generally 0.125 to 1 mg/ml. Reactions were initiated by addition of the UGT enzyme. Termination of the reaction was achieved by the addition of 0.2 ml of ethanol containing 2% ascorbic acid. Samples were centrifuged for 10 min (10,000g) to remove protein, and 140 or 150 µl of supernatant was injected onto HPLC. Reactions were carried out in reduced light in amber Eppendorf tubes due to the light sensitivity of bilirubin. Control incubations contained all the assay components minus the UDPGA cofactor. Under these conditions, percent metabolism was less than 10%. The recovery of the glucuronides in the supernatant was quantitative as determined by extraction of the product pellet.

For the time course experiment, incubations containing 1.25 mg/ml protein and 10 µM bilirubin were terminated after 10, 20, 30, and 40 min. For protein concentration dependence experiment, the final protein concentrations were 0.33, 1.0, 1.67, and 2.3 mg/ml in incubations containing 50 µM bilirubin, and the reactions were terminated after 40 min. For substrate concentration-dependent experiments, the bilirubin concentrations were 2, 5, 10, 20, 30, and 60 µM in incubations containing a final protein concentration of 1.67 mg/ml, and the reactions were terminated after 35 min.

Incubation Conditions with Other UGT Enzymes. Table 1 shows the substrates and inhibitors used for assaying UGT activities. In general, incubation mixtures contained 50 mM buffer, 2 mM UDPGA, 10 mM MgCl2 subtype, and enzyme proteins in a final volume of 0.2 ml. Following incubations, the reactions were terminated by adding 0.05 to 0.2 ml of 6% acetic acid in acetonitrile. After removal of protein by centrifugation for 10 min (10,000g), the supernatant was injected onto HPLC.

Determination of IC50 and Kd. Incubations were performed in duplicate for IC50 determinations. The test substance concentrations were 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 µM. For atazanavir, the final bilirubin concentration was 8 µM, the assay time was 40 min, and the final protein concentration was 1 mg/ml. For indinavir, the final bilirubin concentration was 5 µM, the assay time was 35 min, and the final protein concentration was 1 mg/ml.

Incubations were performed in triplicate for Kd determinations. For assays with atazanavir, the bilirubin concentrations were 3, 6, 9, 12, and 15 µM, and the atazanavir concentrations were 0, 3, 6, and 12 µM. Incubations contained 1 mg/ml protein and were run for 38 min. For indinavir, the bilirubin concentrations were 2, 5, 10, and 20 µM, and the indinavir concentrations were 0, 50, 100, 150, and 250 µM. Incubations contained 1 mg/ml protein and were run for 35 min.

HPLC Method. For analyzing bilirubin glucuronidation incubations, HPLC conditions consisted of two mobile phases: 0.1% trifluoroacetic acid in H2O (A) and 0.1% trifluoroacetic acid in 100% acetonitrile (B). Initial conditions consisted of 90% A and 10% B. Bilirubin glucuronidates were separated at 45°C by a linear increase of solvent B from 10 to 100% over 25 min and held at 100% B for 5 min before returning to initial conditions. The flow rate was 1 ml/min. The HPLC column was a C18 LiChrospher (5 µM, 4 × 25 mm; Merck, Whitehouse Station, NJ). Bilirubin and glucuronide metabolites were detected at 450 nm. In the presence of expressed UGT1A1 and UDPGA, bilirubin was glucuronidated to both the cluster of four peaks for monoglucuronides (15.4–16.6 min) and a diglucuronide product (12.5 min) (Figs. 2 and 3). The retention times and number of bilirubin glucuronide isomers (cis-trans- and positional isomers) detected in the current study are in good agreement with a previous report (Odell et al., 1990). Bilirubin glucuronide peaks were quantitated using bilirubin in a buffer (solvent A and B, 50:50, v/v) as a standard because bilirubin glucuronide metabolites are not commercially available. The sum of the peak area for the mono- and diglucuronides, including two minor isomers, were used for quantitation of total bilirubin glucuronidation.

The supernatant of UGT1A3 reaction was injected on a 4.6 × 250 mm, 5 µM, C18 Zorbax HPLC column (Agilent Technologies, Palo Alto, CA) and separated at 45°C at a flow rate of 1 ml/min with a mobile phase of 10% menthol (A), 100% menthol (B), and 1 mM perchloric acid in 30% acetonitrile in water (C). Initial conditions were 85% A and 15% C. Mobile phase B was increased linearly from 0 to 85% over 20 min while keeping C at 15% and held at 85% B for 2 min before returning to initial conditions. The column was allowed to equilibrate for 11 min before the next injection. The absorbance of the product was measured at 280 nm, and the response was quantitated by comparison to a standard curve of 17β-estradiol 3-glucuronide.

The supernatant of UGT1A6, UGT1A9, and UGT2B7 reactions was injected on a 4.6 × 250 mm, 5 µM, C18 Zorbax HPLC column and separated at 45°C at a flow rate of 1 ml/min with a mobile phase of 10% methanol (A), 100% methanol (B), and 1 mM perchloric acid in 30% acetonitrile in water (C). Initial conditions were 80% A, 10% B, and 10% C. Mobile phase B was increased linearly from 10 to 90% over 15 min while keeping C at 10% and returned to initial conditions over 10 s. The column was allowed to equilibrate for 15 min before the next injection. The absorbance of the product 4-trifluoromethyl-7-hydroxyxoumarin glucuronide was measured at 325 nm, and the response was quantitated by comparison to a standard curve of 4-trifluoromethyl-7-hydroxyxoumarin glucuronide.

The supernatant from the UGT1A4 and trifluoperazine reaction was injected on a 4.6 × 250 mm, 5 µM, C18 Zorbax HPLC column and separated at 45°C with a mobile phase of 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. Initial conditions consisted of 70% A and 30% B. Mobile phase B was increased to 51% by a linear gradient over 14 min and then returned to initial conditions over 10 s, and the column was re-equilibrated for 10 min before the next injection. The absorbance of the product was measured at 256 nm, and the response was quantitated by comparison to a standard curve of trifluoperazine. The parent trifluoperazine was used as the quantitation standard because the authentic metabolite trifluoperazine glucuronide was not available commercially.

Data Analysis. The IC50 value was determined by linear interpolation. The mechanism of inhibition (i.e., competitive, noncompetitive, uncompetitive, or
mixed-type) was determined from $1/V$ versus $1/S$ plots at each inhibitor concentration (Segel, 1993). The apparent $K_i$ was determined from the $x$-intercept of a replot of the mean slopes of the double-reciprocal plot versus $[I]$ (Segel, 1993). Likewise, the apparent $\alpha K_i$ was determined from the $x$-intercept of the mean $y$-intercepts of the double-reciprocal plot versus $[I]$ (Segel, 1993). Graphs were created and linear regression and nonlinear regression calculations were carried out using SigmaPlot software (SPSS Inc., Chicago, IL). Statistical analysis [mean, standard deviation, and covariance (CV)] were determined using Microsoft Excel software.

Rowland and Matin (1973) developed an equation, $\frac{AUC_{i}}{AUC} = 1 + \frac{[I]}{K_i}$, to predict the increase in drug AUC for oral or intravenous drugs caused by a competitive inhibitor, where $[I]$ is the inhibitor concentration at the enzyme site that is available to the metabolism enzyme, and $K_i$ is the in vitro inhibition constant (Ito et al., 1998; Yao and Levy, 2002). This approach is only valid when a drug’s clearance is mainly mediated by one metabolic enzyme. This in vitro-in vivo scaling approach is also applicable to noncompetitive inhibition when the substrate concentration is much smaller than its $K_m$, which is true for most clinical situations (Ito et al., 1998). The inhibition of bilirubin glucuronidation by HIV protease inhibitors would not be mechanism-based since they are not even UGT enzyme substrates; it is highly unlikely to be uncompetitive since very few of examples of uncompetitive inhibition are known, and it is likely that the inhibition follows a competitive, noncompetitive, or a mixed model. The apparent $K_i$ values for competitive portion of the inhibition were determined for atazanavir and indinavir. When the assays are performed at a substrate concentration equivalent to the $K_m$ value, $K_i = 1/2 IC_{50}$ for competitive inhibition and $K_i = IC_{50}$ for noncompetitive inhibition. The inhibition type has not been elucidated for lopinavir, nelfinavir, ritonavir, and saquinavir and so $K_i = 1/2 IC_{50}$ for competitive inhibition was used for these inhibitors. Using a competitive inhibition model is a conservative approach to minimize false-negative predictions since the lowest possible estimate of $K_i$ is used.
**Results**

The glucuronidation of bilirubin was shown to be linear for at least 50 min under the incubation conditions used. The rate of glucuronidation increased linearly with increasing amounts of proteins up to 2.3 mg/ml (the highest protein concentration used). Bilirubin glucuronidation appeared to follow simple Michaelis-Menten kinetics. A non-linear regression/direct plot analysis resulted in an apparent Km and Vmax of 4 μM and 80 pmol/min/mg protein, respectively. The apparent Km value reported in this study was in good agreement with a previous study that demonstrated an apparent Km of about 5 μM for bilirubin glucuronidation by recombinant UGT1A1 (Ciotti et al., 1998). Other studies have reported a higher Km value (25 μM) for bilirubin glucuronidation by the expressed UGT1A1 enzyme (Senafi et al., 1994). The reason for this discrepancy is not known but is likely due to differences in the cell line and assay methodology. Under the same assay conditions, pooled human liver microsomes demonstrated an apparent Km value of 6.2 μM and a Vmax of 1000 pmol/min/mg protein for bilirubin glucuronidation (data not shown). The apparent Km values for the bilirubin glucuronidation were similar between the human cDNA-expressed UGT1A1 and the human liver microsomes.

Table 2 shows the IC50 values found for the inhibition of several UGT enzymes expressed in baculovirus-infected insect cells by the HIV inhibitors atazanavir, indinavir, saquinavir, lopinavir, ritonavir, and nelfinavir. Human UGT1A1 was inhibited by all the protease inhibitors tested, with a potency rank of atazanavir > lopinavir > nelfinavir > saquinavir > ritonavir > indinavir. The inhibition potency rank orders are different for UGT1A3 and UGT1A4. For UGT1A3, lopinavir, saquinavir, ritonavir, and atazanavir had a similar inhibition potential and nelfinavir and indinavir showed a lower inhibition potential. For UGT1A4, the inhibition rank order follows ritonavir > lopinavir > saquinavir > atazanavir > nelfinavir > indinavir. No significant inhibition of UGT1A4 by indinavir and of UGT1A6, 1A9, or 2B7 was observed by any of these HIV protease inhibitors.

The inhibition of bilirubin glucuronidation was further investigated in both human liver microsomes and cDNA-expressed UGT1A1 in lymphoblast cells by selected HIV protease inhibitors. Table 3 shows the IC50 and estimated Ki values for inhibition of bilirubin glucuronidation by these inhibitors. Indinavir demonstrated the highest IC50 value and atazanavir the lowest IC50 value in human liver microsomes and expressed UGT1A1. The IC50 values in human liver microsomes were similar to those expressed UGT1A1 for atazanavir, indinavir, and saquinavir. Nelfinavir demonstrated a significantly lower IC50 value in human liver microsomes than in the expressed UGT1A1, which was confirmed with additional experiments (data not shown).

Atazanavir was shown to be an inhibitor of bilirubin glucuronida-

<table>
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<tr>
<th>HIV Inhibitor</th>
<th>UGT1A1</th>
<th>UGT1A3</th>
<th>UGT1A4</th>
<th>UGT1A6</th>
<th>UGT1A9</th>
<th>UGT2B7</th>
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<tbody>
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<td>2.3</td>
<td>7.9</td>
<td>21</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>26</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>8.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>3.9</td>
<td>7.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Ritonavir</td>
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<td>6.3</td>
<td>2.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Nelfinavir</td>
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<td>60</td>
<td>55</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
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</table>

a Human cDNA-expressed UGT1A1 in baculovirus-infected insect cells.

b The value was 87 μM from a determination in cDNA-expressed UGT1A1 in lymphoblast cells as described in Table 3. The IC50 value was 111 μM from the determination in cDNA-expressed UGT1A1 in baculovirus-infected insect cells.

Table 3 shows the IC50 values found for the inhibition of several UGT enzymes expressed in baculovirus-infected insect cells by the HIV inhibitors atazanavir, indinavir, saquinavir, lopinavir, ritonavir, and nelfinavir. The inhibition of bilirubin glucuronidation was further investigated in both human liver microsomes and cDNA-expressed UGT1A1 in lymphoblast cells by selected HIV protease inhibitors. Table 3 shows the IC50 and estimated Ki values for inhibition of bilirubin glucuronidation by these inhibitors. Indinavir demonstrated the highest IC50 value and atazanavir the lowest IC50 value in human liver microsomes and expressed UGT1A1. The IC50 values in human liver microsomes were similar to those expressed UGT1A1 for atazanavir, indinavir, and saquinavir. Nelfinavir demonstrated a significantly lower IC50 value in human liver microsomes than in the expressed UGT1A1, which was confirmed with additional experiments (data not shown).

Atazanavir was shown to be an inhibitor of bilirubin glucuronida-

<table>
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<tr>
<th>HIV Inhibitor</th>
<th>IC50 Valuesa</th>
<th>K estimatesb</th>
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<tr>
<td>Atazanavir</td>
<td>2.4</td>
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<tr>
<td>Indinavir</td>
<td>87</td>
<td>68</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>7.3</td>
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</tr>
<tr>
<td>Nelfinavir</td>
<td>8.4</td>
<td>4.2</td>
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</table>

a Human cDNA-expressed UGT1A1 in lymphoblast cells.

b Positive control was β-estradiol (UGT1A1).

c The IC50 value was estimated based on Ki = 1/2 × IC50 when [S] = Km, assuming a competitive inhibition.
mated from IC\textsubscript{50} values for other drugs with an assumption of a competitive inhibition. There is no general correlation when C\textsubscript{max} or C\textsubscript{min} were used directly, and the C\textsubscript{max}/K\textsubscript{i} scaling produced false positive for lopinavir, ritonavir, and nelfinavir. However, when a free or unbound concentration, C\textsubscript{max,u}, was used, the intrinsic fraction of UGT1A1 that can be inhibited (I/K\textsubscript{i}) was 0.1 for atazanavir and indinavir, which are known to produce hyperbilirubinemia in some patients and was <0.1 for saquinavir, lopinavir, ritonavir, and nelfinavir, which are not known to produce hyperbilirubinemia. Saquinavir, lopinavir, ritonavir, and nelfinavir have a K\textsubscript{i} value of about 5- to 10-fold lower than indinavir, but their unbound fractions were more than 20-fold lower than indinavir, resulting in lower C\textsubscript{max,u}/K\textsubscript{i} values than indinavir. C\textsubscript{min,u}/K\textsubscript{i} values were too low to be meaningful.

**Discussion**

Our results have demonstrated that atazanavir, indinavir, saquinavir, lopinavir, ritonavir, and nelfinavir inhibited the enzyme activity of UGT1A1, UGT1A3, and UGT1A4, and had little effect on UGT1A6, UGT1A9, and UGT2B7. This selectivity profile is not unexpected based on amino acid sequence analysis of the UGT enzymes (Tukey).

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**Fig. 4.** A, Lineweaver-Burke plot (1/V versus 1/[S]) of substrate concentration dependence of bilirubin glucuronidation in the presence of fixed concentrations of atazanavir (inhibitor). Bilirubin concentrations were 3, 6, 9, 12, and 15 μM. B, replot of the slopes and y-intercepts from the double-reciprocal plot versus the inhibitor atazanavir. The x-intercept of the slope replot is equal to −K\textsubscript{i} (1.9 μM). The x-intercept of the y-intercept replot is equal to −αK\textsubscript{i} (16.4 μM).
Summary of inhibition kinetics of UGT1A1 bilirubin glucuronidation by atazanavir and indinavir.

\[
\begin{align*}
E + S & \rightleftharpoons ES \rightarrow E + P \\
V & = \frac{V_{\text{max}}[S]}{K_m(1 + [I]/K_i) + [S](1 + [I]/\alpha K_i)}
\end{align*}
\]

where \(E\) = UGT; \(S\) = bilirubin; and \(I\) = BMS-232632 or indinavir.

<table>
<thead>
<tr>
<th>HIV Inhibitor</th>
<th>(K_i)</th>
<th>(\alpha K_i)</th>
<th>Inhibition Mechanism</th>
<th>Predicted Inhibition at (C_{ss})</th>
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<tr>
<td>Atazanavir*</td>
<td>1.9</td>
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<td>47.9</td>
<td>1317 ((\alpha = 27.5))</td>
<td>Linear mixed†</td>
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* Human cDNA-expressed UGT1A1 in lymphoblast cells.
† UGT1A1: apparent \(K_m = 4\) \(\mu M\) and \(V_{\text{max}} = 80\) pmol/min mg protein in this set of experiments.
‡ A noncompetitive mechanism with a \(K_i = 100\) \(\mu M\) was reported (Food and Drug Administration summary basis of approval, indinavir).
§ For atazanavir, \(C_{ss} = 1.73\) \(\mu M\) (400 mg q.i.d.); for indinavir, \(C_{ss} = 3.84\) \(\mu M\) (Crixivan package insert, 800 mg i.d.); [bilirubin] = 6.84 \(\mu M\).

and Strassburg, 2000). UGT1A3 and UGT1A4 share 93% identity of amino acid sequences, and they both share 71% homology with UGT1A1. The amino acid sequence homology is decreased to 66% with UGT1A6 and UGT1A9 and to 41% with UGT2B7. The close amino acid sequences between UGT1A1, UGT1A3, and UGT1A4 may generate a similar three-dimensional structure for binding to these structurally related HIV protease inhibitors. UGT1A3 and UGT1A4 catalyze the glucuronidation of important endogenous substances including primary, secondary, and tertiary amines and steroids (Green et al., 1998). Although implications of the in vitro inhibition of UGT1A3 and UGT1A4 are currently not known, our inhibition data suggest that HIV protease inhibitors would be useful inhibitors to study UGT1A3 and UGT1A4 enzymes. The following discussion will focus on inhibition of UGT1A1 and the subsequent effects on bilirubin glucuronidation.

The most abundant naturally occurring bilirubin structure is designated bilirubin-4Z,15Z-IXa. Its stereochemical configuration favors formation of internal hydrogen bonds between the propionic acid chains and the polar –NH–CO– and =NH groups (lactam and imino groups) on the opposite half of the molecule. The hydrogen bonds actually fix the molecule in a rigid three-dimensional configuration that blocks exposure of the polar groups and leads to bilirubin’s hydrophobic properties (Berk et al., 1974). The fact that bilirubin is also a potent inhibitor of HIV-1 protease (McPhee et al., 1996) suggests that these HIV protease inhibitors may share structural similarities to bilirubin. Although these protease inhibitors are poor substrates for human UGT enzymes, they do seem to bind to the substrate binding site of UGT1A1 since our data support that atazanavir and indinavir were at least partially competitive inhibitors of UGT1A1.

The exposure of a drug may be increased if its major clearance pathways are inhibited by a second compound. Drug-drug interactions through inhibition of P450 or transporter-mediated clearance are a major concern because they can lead to dramatic alteration of a drug’s efficacy or safety profiles. In contrast to P450-mediated pathways, for drugs primarily cleared by glucuronidation there are only a few reports describing clinically relevant drug interactions through inhibition of UGT enzymes, and the increase in drug exposures are typically less than 2-fold in the presence of UGT inhibitors (Liston et al., 2001; Boase and Miners, 2002; Lin and Wong, 2002; Williams et al., 2004). The less than 2-fold exposure increase for drugs primarily
cleared by glucuronidation has been attributed to the relatively high $K_m$ values for UGT substrates compared with liver drug concentrations at therapeutic doses and the fact that multiple UGT enzymes are often involved in the metabolism of a single substrate (Williams et al., 2004; Kiang et al., 2005).

The inhibition of an endogenous metabolic pathway such as UGT1A1-mediated bilirubin glucuronidation by indinavir and atazanavir can be viewed mechanistically as analogous to a drug-drug interaction. Although most of the drugs that use enzymes as disease targets inhibit formation of enzyme products for their pharmacological activities such as the inhibition of cleavage of the HIV gal-pol polyprotein into the functional subunits by the protease inhibitors, very few cases of inhibition of endogenous metabolic pathways have been reported to associate with accumulation of the enzyme substrates.

**Fig. 6.** A, Lineweaver-Burke plot ($1/V$ versus $1/[S]$) of bilirubin glucuronidation in the presence of increasing concentrations of indinavir. Bilirubin concentrations were 2, 5, 10, and 20 μM. B, replot of the slopes and y-intercepts from the double-reciprocal plot versus the inhibitor indinavir. The $x$-intercept ($K_i$) of slope versus $[I]$ equals 47.9 μM. The $x$-intercept ($αK_i$) of y-intercept versus $[I]$ equals 1317 μM.
leading to toxicities. Drugs have the potential to inhibit a metabolic reaction leading to elevation of the level of the endogenous substance. The hyperbilirubinemia associated with administration of indinavir and atazanavir may thus be reasonably expected to be mediated by inhibition of UGT1A1 as described herein. Consistent with this mechanism, unconjugated serum hyperbilirubinemia was significantly higher in Gilbert patients treated with either atazanavir or indinavir compared with untreated Gilbert patients who have a lower base levels of UGT1A1 activities than normal subjects (O’Mara et al., 2000; Zucker et al., 2001). The unconjugated hyperbilirubinemia associated with administration of indinavir and atazanavir was rapidly reversible and asymptomatic, and not associated with elevations of hepatic enzymes such as alanine transaminase or aspartate transaminase (Sulkowski, 2004). In addition to UGT1A1 inhibition, indinavir was recently shown to inhibit OATP1B1, a hepatic uptake transporter for bilirubin, which provides an additional potential mechanism for indinavir-induced hyperbilirubinemia (Campbell et al., 2005). In the same report, saquinavir did not inhibit OATP1B1, which correlates with the fact that saquinavir does not produce hyperbilirubinemia. It is not known whether atazanavir inhibits hepatic OATP1B1.

The \([I/K_i]\) approach has been used to predict or correlate many in vivo drug-drug interactions mediated by P450 enzymes with the use of in vitro \(K_i\) data and some measure of in vivo inhibitor concentrations (Bertz and Grannerman, 1997; Ito et al., 1998; Thummel and Bertz, 1997; Ito et al., 1998; Thummel and Bertz, 1998). To be practical and to avoid false negatives caused by underestimation of the unbound drug concentrations, the maximum plasma unbound concentration \(C_{\text{max},u}\) is used for our estimation, which still would likely underestimate the concentration at the entrance to the liver where both blood flow from the hepatic artery and portal vein from gastrointestinal absorption contribute (Ito et al., 2002).

An elegant study by Rhame et al. (2004) demonstrated that \(C_{\text{min}}\) may also contribute to hyperbilirubinemia induced by indinavir. A dose of indinavir at 667 mg in combination with ritonavir at 100 mg every 12 h produced a 6-fold increase in indinavir \(C_{\text{min}}\) (from 0.25 to 1.5 \(\mu\)M) as well as a 1.6-fold increase in indinavir AUC\(_{0-24}\) compared with indinavir alone at 800 mg every 8 h. The indinavir \(C_{\text{min}}\) level of these two dosing regimens were comparable.

### Table 5: Relationship of UGT1A1 inhibition and occurrence of hyperbilirubinemia by HIV protease inhibitors

| HIV Inhibitor | Dose | \(F_u^*\) | \(C_{\text{max}} - C_{\text{min}}\) | \(C_{\text{max},u} - C_{\text{min},u}\) | \(K_i\) | \(C_{\text{max}}/K_i - C_{\text{min}}/K_i\) | \(C_{\text{max},K_i} - C_{\text{min},K_i}\) | Observed Hyperbilirubinemia
<table>
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</thead>
<tbody>
<tr>
<td>Atazanavir</td>
<td>400 q.i.d.</td>
<td>0.135</td>
<td>6.28–0.312</td>
<td>0.847–0.042</td>
<td>1.9</td>
<td>3.31–0.16</td>
<td>0.45–0.02</td>
<td>Yes, some patients</td>
</tr>
<tr>
<td>Indinavir</td>
<td>800 t.i.d.</td>
<td>0.40</td>
<td>12.6–0.293</td>
<td>5.04–0.117</td>
<td>47.9</td>
<td>0.26–0.06</td>
<td>0.11–0.002</td>
<td>Yes, some patients</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>600 t.i.d.</td>
<td>&lt;0.02</td>
<td>0.29–0.060</td>
<td>0.006–0.001</td>
<td>6.52</td>
<td>0.04–0.01</td>
<td>0.04–0.01</td>
<td>No</td>
</tr>
<tr>
<td>Lopinavir*</td>
<td>400 b.i.d.</td>
<td>&lt;0.02</td>
<td>15.3–0.837</td>
<td>0.306–0.175</td>
<td>4.3</td>
<td>3.56–2.03</td>
<td>0.07–0.04</td>
<td>No</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>700 t.i.d.</td>
<td>&lt;0.02</td>
<td>15.6–5.15</td>
<td>0.312–0.103</td>
<td>9.5</td>
<td>1.4–0.54</td>
<td>0.03–0.01</td>
<td>No</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>750 t.i.d.</td>
<td>&lt;0.02</td>
<td>6.74–2.44</td>
<td>0.134–0.049</td>
<td>5.5</td>
<td>0.22–0.44</td>
<td>0.02–0.01</td>
<td>No</td>
</tr>
</tbody>
</table>

* The combination of lopinavir and ritonavir (400 mg/100 mg) was approved for clinical use (Hurst and Faulds, 2000).

1 Plasma protein unbound drug fraction \(F_u\), maximum plasma drug concentration \((C_{\text{max}})\), and plasma drug concentration at trough \((C_{\text{min}})\) values were from literature reports (Flexner, 2000; de Maat et al., 2003; Goldsmith and Perry, 2003). Free drug concentrations were calculated by multiplying drug concentrations by \(F_u\).

2 \(K_i\) was estimated based on \(K_i = 1/2 < C_{\text{min}}\) when \(F_u\) < 0.5 assuming a competitive inhibition.

3 \(AUC_{\text{d}}/AUC = 1 + [I]/K_i\) (Ito et al., 1998); \([I] = C_{\text{max}}\) for prediction.

4 Elevated unconjugated bilirubin was associated with administration of atazanavir and indinavir in some patients (Flexner, 2000; Goldsmith and Perry, 2003).

There are additional factors that could confound our capability to predict the magnitude of unconjugated bilirubin elevation from in vitro data. Many of these would be expected to be similar to those encountered when attempting to predict drug-drug interactions resulting from P450 inhibition, including selection of the most appropriate measure of in vivo inhibitor concentration (free drug versus total drug and different inhibitor concentrations at the enzyme active site from in...
the circulation) and corrections of in vitro parameters due to microsomal binding. These factors have been the subject of extensive reviews (Bertz and Granner, 1997; Ito et al., 1998; Thummler and Wilkinson, 1998). The prediction of interactions caused by inhibition of UGT enzymes involves several additional variables (Lin and Wong, 2002; Kiang et al., 2005). These are associated with technical limitations of in vitro glucuronidation experiments to determine Ki, and may explain why the quantitative extrapolations from in vitro data to the in vivo observations remain to be validated (Remmell, 2002). For UGT-mediated glucuronidation reactions, Kiang et al. (2005) described many cases in which potent in vitro inhibition of UGT enzymes did not correlate with in vivo effects and suggested to take cautions when using in vitro inhibition data. The in vitro experiments typically require disruption of the endoplasmic reticulum membrane by pore-forming molecules such as alamethicin to increase access of substrate and cofactor and to facilitate removal of metabolite and UDP from the lumenerally localized UGT-active site (Fisher et al., 2001).

Nonspecific binding to proteins and phospholipids has been demonstrated to decrease inhibition potency (increased apparent Ki) (Margolis and Obach, 2003). It has been reported that UGT1A1 displays allosteric binding properties for substrates and inhibitors (Rios and Tephy, 2002; Williams et al., 2002). Collectively, use of a fixed and projected inhibitor concentration, the potentially allosteric properties of UGT1A1, and sensitivity toward lipid microenvironment on bilirubin glucuronidation (Whitmer et al., 1986) may have limited our quantitative prediction of reversible hyperbilirubinemia from in vitro UGT1A1 inhibition by atazanavir and indinavir.

In summary, our in vitro data demonstrate that both atazanavir and indinavir inhibit UGT1A1-mediated bilirubin glucuronidation. The results provide a mechanism for the hyperbilirubinemia associated with administration of atazanavir as well as indinavir. We have used a variety of techniques to try to correlate the in vitro UGT1A1 inhibition parameters for a series of HIV protease inhibitors to the clinical observations of hyperbilirubinemia. Our results indicate that the unbound Cmax inhibitor concentration is a better predictor of the clinical observations than the total Cmax plasma concentration. Future studies will be necessary to determine whether this finding can be extrapolated to other UGT interactions. In addition, more studies are needed to better define how the pharmacokinetics of enzyme inhibitors influence drug-drug interactions, especially in the case of inhibition of endogenous pathways.

Acknowledgments. We thank Dr. Scott Grossman for a number of invaluable suggestions, and Drs. Michael Sinz and Dan Cui for review of the manuscript.

References


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