ATAZANAVIR: EFFECTS ON P-GLYCOPROTEIN TRANSPORT AND CYP3A METABOLISM IN VITRO

Elke S. Perloff, Su X. Duan, Paul R. Skolnik, David J. Greenblatt, and Lisa L. von Moltke

Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine and Tufts-New England Medical Center, Boston, Massachusetts (E.S.P., S.X.D., D.J.G., L.L.v.M.); and Center for HIV Care and Research, Section of Infectious Disease, Boston University School of Medicine and Boston Medical Center, Boston, Massachusetts (P.R.S.)

Received November 4, 2004; accepted March 8, 2005

ABSTRACT:

The effect of atazanavir on P-glycoprotein (P-gp) expression and activity, as well as its inhibitory potency against CYP3A activity, was evaluated in vitro. Induction of P-gp activity and expression was studied using LS180V cells. P-gp inhibition was studied using both LS180V cells and Caco-2 cells. P-gp activity was assessed by measuring P-gp-mediated rhodamine 123 (Rh123) transport, and P-gp expression was determined using SDS-polyacrylamide gel electrophoresis/Western blot analysis. CYP3A inhibition was tested using triazolam hydroxylation in human liver microsomes (HLM). Extended (3-day) exposure of LS180V cells to 30 μM atazanavir caused a 2.5-fold increase in immunoreactive P-gp expression as well as a concentration-dependent decrease of intracellular Rh123 to a mean 45% (S.D. 5.2%) of control. Acute exposure (2 h) of LS180V cells to atazanavir increased intracellular Rh123 concentrations up to 300% of control at 100 μM atazanavir. At 30 μM and above, acute atazanavir exposure reversed P-gp induction caused by 3-day pretreatment with 10 μM ritonavir. P-gp inhibition was also observed in Caco-2 cells, causing an effect comparable to that observed for the known P-gp inhibitor verapamil (50% of control). In HLM, atazanavir was an inhibitor of triazolam hydroxylation, with inhibitory potency greatly increased by preincubation. IC₅₀ values with and without preincubation were 0.31 μM (S.D. 0.13) and 5.7 μM (S.D. 4.1), respectively. Thus, atazanavir is an inhibitor and inducer of P-gp as well as a potent inhibitor of CYP3A in vitro, suggesting a potential for atazanavir to cause drug-drug interactions in vivo.

Atazanavir (BMS-232632, Reyataz) is an azapeptide HIV-1 protease inhibitor recently approved by the Food and Drug Administration for the combination treatment of HIV-1 infection (Piliero, 2002; Goldsmith and Perry, 2003; Musial et al., 2004; Orrick and Steinhardt, 2004). Atazanavir is the seventh addition to the family of HIV protease inhibitors but appears to be more potent than other members of its class. Atazanavir shows high relative selectivity for HIV-1 protease (Robinson et al., 2000) and appears to lack the adverse effects on blood lipid profiles seen with other protease inhibitors, possibly eliminating the need for cholesterol-lowering comedications (Goldsmith and Perry, 2003). Atazanavir is an inhibitor of cholesterol 7 α-hydroxylase (Goldsmith and Perry, 2003). The primary resistance mutation of atazanavir is distinct, with low cross-resistance to other related compounds (Colombo et al., 2004). Similar to other protease inhibitors, atazanavir is extensively metabolized, mainly by CYP3A (Goldsmith and Perry, 2003). Atazanavir has been successfully used in combination therapy in both treatment-naive and treatment-experienced patients (Haas et al., 2003; Havlir and O’Marro, 2004; Squires et al., 2004).

HIV patients are typically treated with multiple other drugs in addition to their antiretroviral regimen. Although these combination drug regimes have substantially improved survival, they are also associated with a large potential for drug-drug interactions (de Maat et al., 2003). Drugs used in HIV treatment therefore need to be evaluated carefully with regard to their effects on hepatic and intestinal metabolizing enzymes (especially CYP3A) as well as their interactions with drug transporters (especially P-glycoprotein; P-gp). Several HIV protease inhibitors as well as non-nucleoside reverse transcriptase inhibitors have already been identified as substrates, inhibitors, or inducers of P-gp and CYP3A (Kim et al., 1998; von Moltke et al., 1998b, 2000, 2001; Profit et al., 1999; Perloff et al., 2000, 2001, 2002, 2003; Störmér et al., 2002).

P-gp-mediated drug transport in vitro has been successfully studied using the human colon adenocarcinoma cell lines Caco-2 (Artursson et al., 1996; Quaroni and Hochman, 1996) and LS180 (Herzog et al., 1993; Schuetz et al., 1996; Perloff et al., 2000; Störmér et al., 2002). Rh123 cell exclusion studies in LS180V cells are especially useful because this model allows the assessment of P-gp induction (activity and expression) and P-gp inhibition in the same cell line (Perloff et al., 2003).

To anticipate drug-drug interactions associated with combination drug therapy, this in vitro study investigated the effects of the HIV protease inhibitor atazanavir on the activity of P-gp-mediated Rh123 transport and CYP3A metabolism.
transport, on P-gp expression, as well as on CYP3A-mediated drug metabolism.

**Materials and Methods**

**Chemicals.** Atazanavir was extracted into methanol from its clinical dosage form. It was assumed that atazanavir was completely extracted from the dosage form. Quantification could not be performed for lack of a pure reference standard. Other drugs and chemicals were purchased from commercial sources or were kindly provided by their pharmaceutical manufacturers.

**Cell Lines.** The human colon adenocarcinoma cell line LS180 (Pinto et al., 1983) was obtained from the American Type Culture Collection (Manassas, VA). Cells were selected for elevated P-gp levels with increasing concentrations of vinblastine (1 to 2 to 4 to 8 ng/ml) and maintained at 4 ng/ml until initiation of induction experiments (similar to Herzog et al., 1993). The vinblastine-selected cell line is denoted LS180V. LS180V cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM pyruvic acid, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 4 mg/ml vinblastine.

The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection and used at passages 20 to 30. Caco-2 cells were grown in MEM supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM pyruvic acid, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

**Induction and Inhibition of Rh123 Transport in LS180V Cells.** LS180V cells were seeded at 1 × 10^5/cm² in 24-well tissue culture cluster plates (Corning; Costar, Cambridge, MA). Cells were grown in a humidified chamber (37°C, 5% CO₂) to 90% confluence (3–4 days), when medium was replaced by vinblastine-free medium containing 1 to 100 μM atazanavir and 0.5% DMSO. The known P-gp inducer rifampin (Perloff et al., 2000, 2001) served as a positive control, and vehicle (medium with 0.5% DMSO) served as a negative control. Experiments were done in duplicate. After 72 h, cells were washed three times with MEM containing 10% calf serum and 0.5% DMSO and supplied with vinblastine-free media with or without inhibitor (100 μM verapamil, 3–100 μM atazanavir). Plates were incubated for 30 min and Rh123 (final concentration 5 μM) was added. Cells were placed in the incubator for 90 min and subsequently washed three times with MEM containing 10% calf serum and 0.5% DMSO. Cells were lysed for 30 min (0.5% deoxycholate, 1% Triton X, 1 mM phenylmethylsulfonyl fluoride in 0.05 M KH2PO4, and Rh123 concentrations in the lysate were quantified by fluorescence measurement at 500/550 nm (excitation/emission).

Determination of protein concentration demonstrated comparable cell density in each well. In similar experiments, LS180V cell morphology was evaluated microscopically (Perloff et al., 2001) to assure cell viability. Furthermore, the Rh123 accumulation methodology verifies cell viability and cell membrane integrity itself, such that cell membrane disruption by potential inhibitors yields results that are highly indicative of toxicity. If the LS180V cell membrane is compromised due to toxicity, the washing procedure during Rh123 accumulation experiments greatly reduces intracellular Rh123 (10-fold less compared with positive control values) and verapamil has no reversal effect.

We have previously observed that induction studies exhibit some variability in verapamil inhibitory potential, whereas relative induction results remain fairly constant. This finding may be due to the multiple steps involved in the experiments. However, the possibility of differential effects on transporters other than P-gp by the study drugs cannot be excluded and may contribute to the variable effects of verapamil on Rh123 accumulation in induced cells. In any case, this should not affect comparisons of Rh123 concentrations between different treatments within the same experiment.

**Induction of P-gp Protein Expression in LS180V Cells.** LS180V cells were seeded at 0.5 × 10^5/cm² in 5-cm round tissue culture dishes (Corning; Costar). Cells were grown in a humidified chamber (37°C, 5% CO₂) to 70% confluence (3 to 4 days), when medium was replaced by vinblastine-free medium containing 3 and 30 μM atazanavir and 0.5% DMSO. The known P-gp inducer rifampin served as a positive control; vehicle (medium with 0.5% DMSO) served as a negative control. Experiments were done in triplicate. After 72 h, cells were lysed (0.5% deoxycholate, 1% Triton X, 1 mM phenylmethylsulfonyl fluoride in 0.05 M KH2PO4), sonicated, and centrifuged. Supernatants were analyzed for protein concentration using the BCA protein assay (Pierce Chemical, Rockford, IL) and stored at −80°C until Western blot analysis. The 72-h exposure time for induction experiments was selected based on time course experiments performed with ritonavir in LS180V cells (Perloff et al., 2000, 2001).

Samples were separated by SDS-polyacrylamide gel electrophoresis on 4 to 15% acrylamide gradient gels (Ready-Gel; Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore Corporation, Bedford, MA). The-170 kDa P-gp protein was detected using the monoclonal mouse anti-human P-gp (C219) primary antibody (Signet Laboratories, Dedham, MA), and a horseradish peroxidase-linked sheep anti-mouse IgG secondary antibody (Amersham Biosciences Inc., Piscataway, NJ) with SuperSignal chemiluminescence reagent (Pierce Chemical). Sample preparation, electrophoresis, and transfer were performed at 4°C. Images were acquired using a Kodak Image Station IS440CF (Eastman Kodak, Rochester, NY). Western blots were quantified using computer image analysis (Kodak 1D Image Analysis Software, v3.6.2; Eastman Kodak). Blots were quantified based on a calibration curve run together with each set of samples, allowing for comparison of relative P-gp contents between samples.

**Inhibition of Rh123 Transport in Caco-2 Cells.** Caco-2 cells were seeded at 1 to 2 × 10^5/cm² in 24-well polycarbonate membrane Transwell plates (0.33-cm diameter, 3-μm pore size) (Corning; Costar). Cells were grown in a humidified chamber (37°C, 5% CO₂) with media changes every 3 to 5 days. Transport experiments were conducted in confluent cells on days 12 to 16 post-seeding. Transepithelial electrical resistance was >300 Ω cm², confirming high monolayer confluence. Methanolic solutions of atazanavir were evaporated to dryness, dissolved in 0.5% DMSO, and reconstituted in Opti-MEM medium (Invitrogen, Carlsbad, CA). Solubility of atazanavir in medium was verified by high-performance liquid chromatography analysis. Peak heights were linear with drug concentration over the concentration ranges used (up to 250 μM). Rh123 was added as a concentrated methanolic solution, with final methanol concentration <0.5%. Medium containing Rh123 (5 μM) was added to the apical (A) or basolateral (B) chamber, with the potential inhibitor present in both chambers. Cells were incubated at 37°C, 5% CO₂ for 180 min, and samples were taken (50 μl and 200 μl, respectively, from chamber A and B) from the chamber initially not containing Rh123. Samples from chamber A were diluted with 150 μl of methanol. Rh123 concentrations were determined as described above.

**CYP3A-Mediated Biotransformation Activity in Vitro.** Healthy liver tissue was obtained from the International Institute for the Advancement of Medicine (Exton, PA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MI), or the National Disease Research Interchange (Philadelphia, PA). The tissue was kept at ~80°C until time of microsome preparation. Microsomes were prepared and stored as described previously (von Moltke et al., 1993).

Triazolam-4-hydroxylation and triazolam-α-hydroxylation were used as index reactions for CYP3A activity in vitro. Incubations were performed as described previously (von Moltke et al., 1998a,b, 2000, 2001; Greenblatt et al., 2003; Weemhoff et al., 2003). In brief, incubation mixtures contained 0.05 M KH2PO4 buffer (pH 7.4 at 25°C), 0.5 mM NADP, 3.75 mM t-isocitric acid, 1 U/ml isocitrate dehydrogenase, and 5 mM Mg²⁺. Final volumes were 250 μl with a microsomal protein concentration of 250 μg/ml and a triazolam concentration of 250 μM. Incubation time was 20 min. Atazanavir and ketoconazole (established as a reversible inhibitor of CYP3A) were evaluated for inhibitory activity. Both were tested for reversible (without preincubation of inhibitor with microsomes) and mechanism-based inhibition (20-min preincubation of inhibitor with buffer, cofactors, and microsomes at 37°C prior to exposure to substrate). The effect of increasing preincubation times (5–20 min) on the inhibitory effects of atazanavir and ketoconazole was also evaluated.

Triazolam and its metabolites α-hydroxy-triazolam and 4-hydroxy-triazolam were quantified by isocratic high-performance liquid chromatography with UV detection at 220 nm (von Moltke et al., 1998a).

**Data Analysis.** For studies of intracellular Rh123 concentrations, immunolabeling protein expression, and Rh123 transport, differences between experimental conditions and the corresponding vehicle control were evaluated using Student’s t test, with p < 0.05 being the boundary for statistical significance. For P50 inhibition studies, IC₅₀ values (inhibitor concentrations corresponding to a 50% decrease in reaction velocity) were generated by nonlinear
regression using SigmaPlot (v8.02; SPSS Inc., Chicago, IL) using methodology described previously (von Moltke et al., 1998b).

Results

Induction of Rh123 Transport Activity by Atazanavir in LS180V Cells. Extended exposure (3 days) of LS180V cells to increasing concentrations of atazanavir reduced intracellular Rh123 accumulation in a concentration-dependent manner to 45% of vehicle control at 30 μM atazanavir (Fig. 1, solid bars). The positive control ritonavir (10 μM) caused a decrease in Rh123 uptake to 32% of control. Addition of the P-gp inhibitor verapamil reversed the effect in both controls and drug-treated cells, increasing accumulation of Rh123 to up to 70% above control values (Fig. 1, open bars).

Induction of P-gp Protein Expression by Atazanavir in LS180V Cells. Atazanavir was also evaluated for its ability to induce P-gp expression in LS180V cells after extended exposure. Western blot analysis of membrane preparations of the exposed cells revealed a concentration-dependent increase in immunoreactive P-gp after 3-day atazanavir exposure. Whereas 3 μM atazanavir did not cause a detectable increase in P-gp expression, at 30 μM atazanavir a 2.5-fold increase in P-gp expression over vehicle control was observed (Fig. 2). This effect was comparable to the 2.3-fold induction caused by 10 μM ritonavir, a known P-gp inducer used as a positive control. These Western blot data correspond well to the Rh123 cell exclusion results for atazanavir showing an inductive effect comparable to that seen with ritonavir. Together, these results indicate that after extended exposure to atazanavir or ritonavir, active Rh123 efflux from the cell is increased as a result of P-gp induction.

Inhibition of Rh123 Transport Activity by Atazanavir in LS180V Cells. Acute exposure (2 h) of LS180V cells to increasing concentrations of atazanavir (3–100 μM) inhibited Rh123 cell exclusion, leading to increased intracellular Rh123 concentrations. The effect was concentration-dependent and ranged from a 1.6-fold increase over control at 3 μM atazanavir to a 3-fold increase at 100 μM atazanavir, an effect comparable to that observed for the known P-gp inhibitor verapamil (Fig. 3, open bars).

An additional measure of P-gp inhibitory potency is the ability of a drug to reverse P-gp induction caused by extended exposure to a known P-gp inducer. In LS180V cells pretreated with the inducer ritonavir (10 μM) for 3 days, the decrease in Rh123 accumulation was
readily reversed by acute (2-h) exposure to 100 μM verapamil. Treatment with increasing concentrations of atazanavir showed the same effect, with lower atazanavir concentrations (3–10 μM) leading to partial reversal of the ritonavir induction and higher atazanavir concentrations resulting in intracellular Rh123 concentrations up to 1.5-fold of those seen in uninduced controls (Fig. 3, solid bars).

**Combined Induction and Inhibition of Rh123 Transport Activity in LS180V Cells.** LS180V cells subjected to 3-day induction by increasing concentrations (0, 3, 10, 30 μM) of atazanavir and ritonavir were subsequently exposed acutely to increasing concentrations (0, 3, 10, 30 μM) of the P-gp inhibitor verapamil. At all inducer concentrations tested, verapamil was able to reverse the decrease in Rh123 accumulation caused by P-gp induction. Increasing concentrations of the inducing agent (azidinavir, Fig. 4a; ritonavir, Fig. 4b) initially required increasing concentrations of verapamil for reversal of the inhibitive effect. The increase in inducer concentration from 10 μM to 30 μM, however, showed little effect on the verapamil concentration required to reverse the effect, suggesting that the induction effect plateaus at about 10 μM for both compounds (Fig. 4).

**Inhibition of Rh123 Transport by Azidinavir in Caco-2 Cells.** Azidinavir was evaluated for its potential to inhibit P-gp-mediated basolateral to apical Rh123 transport across Caco-2 monolayers. Azidinavir (3–100 μM) caused a concentration-dependent reduction of basolateral to apical Rh123 transport, whereas apical to basolateral transport remained unchanged (Fig. 5). At the highest concentration tested (100 μM), azidinavir reduced Rh123 transport to 54% of control, whereas the known P-gp inhibitor verapamil (100 μM) caused a reduction to 42% of control under the same conditions.

**P-gp Modulation by Azidinavir in Relation to the Effects of Other HIV Protease Inhibitors.** At a concentration of 10 μM, induction and inhibition of P-gp activity in LS180V cells by azidinavir was compared with the effects of six other protease inhibitors (ritonavir, saquinavir, indinavir, amprenavir, nelfinavir, and lopinavir) under the same conditions.

**Induction of Rh123 Efflux from LS180V Cells.** The most potent inducers of P-gp activity were ritonavir and nelfinavir, which decreased intracellular Rh123 accumulation to 30% and 32% of control, respectively, at 10 μM. The inhibitive effect associated with extended exposure to azidinavir (53% of control) was comparable to that of lopinavir (56%) and saquinavir (58%). Indinavir showed a slightly weaker effect, with a reduction to only 66% of control values. The observed effects were reversed by acute exposure to the P-gp inhibitor verapamil (100 μM), which restored intracellular Rh123 concentrations to 1.2- to 1.7-fold of control values (Fig. 6). Amprenavir, the seventh HIV protease inhibitor currently available for clinical use, showed an atypical response in this extended exposure experiment, resulting in a 2-fold increase in intracellular Rh123 (data not shown). Increasing the number of MEM washes did not affect the atypical increase in Rh123 concentrations seen with am-
prenavir, which is contradictory to findings from Western blot analysis (Perloff et al., 2000) showing increased protein expression. The finding might be due to incomplete removal of AMP by washing, or irreversible alterations of the P-gp protein during the 72-h exposure, leading to reduced activity.

**Inhibition of Rh123 Efflux from LS180V Cells.** Indinavir was the only HIV protease inhibitor that did not cause any degree of P-gp inhibition, with intracellular Rh123 concentrations comparable to those of vehicle controls, both in noninduced and ritonavir-induced LS180V cells (Fig. 7). Of the remaining six compounds, ritonavir appeared to be the weakest inhibitor. Acute ritonavir treatment (10 μM) caused a 20% increase in intracellular Rh123 concentration in noninduced cells and did not completely reverse the inductive effect caused by chronic ritonavir induction (Fig. 7, solid bars). Amprenavir, lopinavir, nelfinavir, and saquinavir showed a similar degree of inhibition with intracellular Rh123 concentrations of 35% to 55% higher than vehicle control values. All four HIV protease inhibitors were able to reverse the reduction in P-gp activity caused by the preceding ritonavir induction, resulting in Rh123 concentrations similar (95% to 108%) to those seen in vehicle-treated cells (Fig. 7). The most potent inhibitor in this model was amprenavir, which caused a 72% increase in Rh123 concentrations in noninduced cells and reversed ritonavir induction to values substantially above control values (145% of vehicle controls).

**Inhibition of CYP3A-Mediated Metabolism in HLM by Atazanavir.** Atazanavir caused a concentration-dependent inhibition of CYP3A. Without preincubation, the formation of α-hydroxy-triazolam and 4-hydroxy-triazolam from triazolam (substrate concentration 250 μM) in HLM was reduced to 9% of control at an atazanavir concentration of 50 μM (Fig. 8a, solid line), an effect comparable to that of 1 μM ketoconazole (Fig. 8b, solid line). Preincubation of HLM with atazanavir (20 min) substantially increased the inhibitory effect, suggesting that atazanavir inhibition of CYP3A may have a mechanism-based (irreversible) component. As a result, a 10-fold lower atazanavir concentration (5 μM) was required to achieve maximal inhibition (6% of control) of CYP3A activity (Fig. 8a, dashed line).

**FIG. 7. P-gp inhibition by atazanavir in comparison with other HIV protease inhibitors.** Intracellular Rh123 accumulation in LS180V cells after extended (3-day) exposure to atazanavir and other HIV protease inhibitors (10 μM) and reversal of the effect by acute (2-h) exposure to verapamil (100 μM). Solid bars, induction followed by 2-h exposure to vehicle (medium with 0.5% DMSO); open bars, induction followed by 2-h exposure to verapamil (100 μM). Data are means ± S.D. of duplicate samples. Values are fractions of vehicle control. For data depicted by solid bars, * indicates significant difference (p < 0.05) compared with vehicle control. Absolute value of vehicle control was 145 pmol of Rh123/mg protein.

**FIG. 8. Inhibition of CYP3A activity by atazanavir.** Formation rates of α-hydroxy-triazolam from triazolam (250 μM) in human liver microsomes with increasing concentration of inhibitor. a, atazanavir: with 20-min preincubation (0.1–50 μM; dashed line) and without preincubation (1–50 μM; solid line). b, ketoconazole (0.05–1 μM); with 20-min preincubation (dashed line) and without preincubation (solid line). Values are percentage of controls without inhibitors. Data are means ± S.E.M. of four human livers. IC50 values are reported in Table 1.
dashed line). Mean IC\textsubscript{50} values for atazanavir concentration versus triazolam-\(\alpha\)-hydroxylation decreased from 5.7 \(\mu\)M (S.D. 4.1 \(\mu\)M) to 0.31 \(\mu\)M (S.D. 0.13 \(\mu\)M) with preincubation. Preincubation did not affect the inhibition caused by ketoconazole, with IC\textsubscript{50} values of 0.18 \(\mu\)M (S.D. 0.14 \(\mu\)M) and 0.22 \(\mu\)M (S.D. 0.24 \(\mu\)M), respectively (Fig. 8b, dashed line). IC\textsubscript{50} values for atazanavir and ketoconazole versus triazolam-\(\alpha\)-hydroxylation and triazolam-4-hydroxylation in four human livers are reported in Table 1.

Additionally, the effect of increasing preincubation times for HLM with and without inhibitor was evaluated in one human liver. Preincubation of HLM with cofactor alone at 37°C (20 min) had no effect on the inhibition of CYP3A activity by atazanavir or ketoconazole (Fig. 9a). Preincubation of HLM with atazanavir, however, led to an increase in inhibitory effect with increasing preincubation time (Fig. 9b), consistent with the possibility that atazanavir acts as an irreversible (mechanism-based) inhibitor of CYP3A. No such effect was seen with the known reversible CYP3A inhibitor ketoconazole.

### Discussion

The present in vitro study showed that atazanavir could significantly modulate P-glycoprotein activity and expression, based on transport of the index substrate Rh123 in cell culture. Additionally, atazanavir was shown to be an inhibitor of CYP3A activity in human liver microsomes. Inhibitory potency was increased by preincubation, consistent with mechanism-based inhibition, as is the case for a number of HIV protease inhibitors including ritonavir and lopinavir (von Molke et al., 2000; Weemhoff et al., 2003).

Expression of transporters other than P-gp, including MRP and dipeptide transporters (Li et al., 2003) in Caco-2 cells, require the use of an index substrate that is relatively specific for the transporter studied. The fluorescent dye Rh123 is frequently used as a P-gp index substrate. A drug screen by the National Cancer Institute in 58 different cell lines showed a good correlation of Rh123 transport with P-gp expression, whereas no correlation was found with the expression of MRP in these cell lines (Lee et al., 1994). Experiments with our Caco-2 cell line (data not shown) demonstrated that 2 mM probenecid and 100 \(\mu\)M indomethacin, both known inhibitors of MRP1, did not inhibit Rh123 transport, thus eliminating MRP1 as a contributing factor to Rh123 transport. In LS180V cells, the effects of P-gp induction (extended exposure to 10 \(\mu\)M ritonavir) and P-gp inhibition (acute exposure to 100 \(\mu\)M verapamil) were comparable between Rh123 and diethyloxacarbocyanine iodide, a compound that is a substrate of P-gp but not MRP (Minderman et al., 1996).

### Table 1

<table>
<thead>
<tr>
<th>Triazolam-(\alpha)-hydroxylation</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir without preincubation</td>
<td>5.11</td>
</tr>
<tr>
<td>with 20-min preincubation</td>
<td>1.16</td>
</tr>
<tr>
<td>Ketoconazole without preincubation</td>
<td>0.22</td>
</tr>
<tr>
<td>with 20-min preincubation</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triazolam-4-hydroxylation</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir without preincubation</td>
<td>6.88</td>
</tr>
<tr>
<td>with 20-min preincubation</td>
<td>1.93</td>
</tr>
<tr>
<td>Ketoconazole without preincubation</td>
<td>0.12</td>
</tr>
<tr>
<td>with 20-min preincubation</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Both inductive and inhibitory effects of atazanavir on Rh123 transport in vitro were concentration-dependent in the range of 3 to 100 \(\mu\)M. The inhibitory effect of atazanavir on triazolam hydroxylation occurred at 10-fold lower concentrations, with a mean IC\textsubscript{50} value of 0.3 \(\mu\)M for mechanism-based inhibition. Since antiretroviral drugs are used in combination therapy, the effect of atazanavir inhibitors on LS180V cells pretreated with a P-gp inducer (10 \(\mu\)M ritonavir) was also tested. Atazanavir increased intracellular Rh123 accumulation and reversed the inhibitory effect caused by extended ritonavir exposure.

Peak plasma concentrations of atazanavir are usually in the range of 2.9 to 5.9 \(\mu\)g/l (approximately 4–8 \(\mu\)M) (Goldsmith and Perry, 2003), and concentrations in the intestine after oral administration of therapeutic atazanavir doses (400 mg) will substantially exceed plasma concentrations and may reach millimolar levels. Considering those in vivo atazanavir concentrations, these data suggest a potential for atazanavir to cause drug interactions at the level of both P-gp-mediated drug transport and enteric CYP3A-mediated drug metabolism.
Plasma concentrations of atazanavir are likely to be sufficient to cause inhibition of CYP3A-mediated hepatic metabolism as well; however, the effects of atazanavir on P-gp activity are probably limited to the intestine, where drug concentrations are higher. The inhibition of P-gp transport and CYP3A metabolism would explain the recent finding that addition of atazanavir to a regimen of saquinavir and ritonavir resulted in a 60% and 40% increase of saquinavir and ritonavir AUC values, respectively (Boffito et al., 2004). Compared with the effects of the other six HIV protease inhibitors on P-gp activity in LS180V cells, atazanavir appears to be comparable to saquinavir and lopinavir with respect to both induction and inhibition of Rh123 transport in LS180V cells. Although no previous data are available for atazanavir, P-gp induction and inhibition have been reported for other protease inhibitors (Profit et al., 1999; Perloff et al., 2000, 2001, 2002; Vishnudvathan et al., 2003). IC50 values have been reported as 6.7 μM for ritonavir (Perloff et al., 2002) and 1.7μM for lopinavir (Vishnudvathan et al., 2003) in Caco-2 cells, indicating more potent P-gp inhibition. Profit et al. (1999) tested four protease inhibitors for P-gp inhibition and found inducer to have no effect, nefinavir to cause moderate inhibition, and saquinavir and ritonavir to be potent inhibitors of Rh123 transport in Caco-2 cells. In our LS180V cell model, we found similar relative inhibition by inducer (none), nefinavir (moderate), and saquinavir (potent); however, ritonavir appears to be only a moderate inhibitor in this model. It should be noted that although the effects of atazanavir in this study fell within the range observed for other HIV protease inhibitors, unexpected yet clinically important direct interactions associated with atazanavir cannot be excluded.

Although several clinical studies have investigated the effects of comedications on atazanavir pharmacokinetics (Goldsmith and Perry, 2003), little information is available on the effects of atazanavir on the pharmacokinetics of other drugs. Studies investigating the potential for P450 induction by atazanavir would provide additional information about its potential for causing drug interactions, since concomitant induction and inhibition of P-gp and P450 may result in dose-dependent as well as time-dependent clinical effects. Additionally, a potential interplay has been suggested between metabolic enzymes and transporters that could confound in vitro predictions of drug interactions (Benet et al., 2003). Therefore, clinical drug interaction studies testing atazanavir together with drugs that are selective substrates of P-gp and CYP3A, respectively, are needed to evaluate the clinical relevance of these in vitro findings.

In conclusion, atazanavir is capable of altering both P-gp and CYP3A activity in vitro. Atazanavir induces P-gp activity and expression, inhibits P-gp-mediated Rh123 transport, and acts as a potent mechanism-based inhibitor of CYP3A activity at clinically relevant concentrations.

References
Vishnudvathan et al. (2003). IC50 values have been reported as 6.7 μM for ritonavir (Perloff et al., 2002) and 1.7μM for lopinavir (Vishnudvathan et al., 2003) in Caco-2 cells, indicating more potent P-gp inhibition. Profit et al. (1999) tested four protease inhibitors for P-gp inhibition and found inducer to have no effect, nefinavir to cause moderate inhibition, and saquinavir and ritonavir to be potent inhibitors of Rh123 transport in Caco-2 cells. In our LS180V cell model, we found similar relative inhibition by inducer (none), nefinavir (moderate), and saquinavir (potent); however, ritonavir appears to be only a moderate inhibitor in this model. It should be noted that although the effects of atazanavir in this study fell within the range observed for other HIV protease inhibitors, unexpected yet clinically important direct interactions associated with atazanavir cannot be excluded.

Although several clinical studies have investigated the effects of comedications on atazanavir pharmacokinetics (Goldsmith and Perry, 2003), little information is available on the effects of atazanavir on the pharmacokinetics of other drugs. Studies investigating the potential for P450 induction by atazanavir would provide additional information about its potential for causing drug interactions, since concomitant induction and inhibition of P-gp and P450 may result in dose-dependent as well as time-dependent clinical effects. Additionally, a potential interplay has been suggested between metabolic enzymes and transporters that could confound in vitro predictions of drug interactions (Benet et al., 2003). Therefore, clinical drug interaction studies testing atazanavir together with drugs that are selective substrates of P-gp and CYP3A, respectively, are needed to evaluate the clinical relevance of these in vitro findings.

In conclusion, atazanavir is capable of altering both P-gp and CYP3A activity in vitro. Atazanavir induces P-gp activity and expression, inhibits P-gp-mediated Rh123 transport, and acts as a potent mechanism-based inhibitor of CYP3A activity at clinically relevant concentrations.

Address correspondence to: Dr. David J. Greenblatt, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. E-mail: dlj.greenblatt@tufts.edu