Influence of atazanavir 200 mg on the intracellular and plasma pharmacokinetics of saquinavir and ritonavir 1600/100 mg administered once daily in HIV-infected patients

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Objectives: To examine cellular and plasma concentrations of atazanavir when given in combination with saquinavir/ritonavir in HIV+ patients.

Methods: Twelve HIV+ patients were receiving saquinavir/atazanavir/ritonavir 1600/200/100 mg once daily and venous blood samples were taken to determine cellular and plasma concentrations of each protease inhibitor at 2, 6, 12 and 24 h. Peripheral blood mononuclear cells were separated by density gradient centrifugation. The ratio of the cellular AUC0–24/plasma AUC0–24 was calculated to determine cellular drug accumulation. Lymphocyte P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP1) expression was determined by flow cytometry. Nine of the patients had previously received a regimen of saquinavir/ritonavir 1600/100 mg; therefore the effect of atazanavir on the cellular and plasma pharmacokinetics of saquinavir and ritonavir was examined.

Results: In vivo cellular and plasma determinations of saquinavir, atazanavir and ritonavir gave accumulation ratios of 4.9, 1.2 and 1.7, respectively. There was no relationship between saquinavir, atazanavir or ritonavir accumulation and P-gp, MRP1 or BCRP expression. When comparing pharmacokinetic values in the nine patients receiving saquinavir/ritonavir with and without atazanavir, the median cellular saquinavir AUC0–24 was significantly increased (34.9–117.2 mg·h/L) on addition of atazanavir (P = 0.004). The C24 of saquinavir in plasma and cells was significantly higher with atazanavir (plasma C24 0.05 versus 0.14 mg/L with atazanavir; cellular C24 0.61 versus 2.03 mg/L with atazanavir, P = 0.02).

Conclusions: The mechanism of differential intracellular protease inhibitor accumulation is unclear. Co-administration of atazanavir caused an increase in both the plasma and cellular exposure (AUC0–24) and C24 of saquinavir but not ritonavir.

Keywords: protease inhibitors, intracellular pharmacokinetics, antivirals

Introduction

The availability of new antiretroviral drugs is critical to provide new treatment options for experienced patients. The azapeptide protease inhibitor (PI) atazanavir, in combination with low dose ritonavir, has been approved for use in experienced HIV patients in Europe. To date, clinical studies have shown that atazanavir has a favourable pharmacokinetic, lipid1,2 and resistance profile3 in comparison with other PIs. Clinical studies are in progress to evaluate the optimal antiretroviral combination with atazanavir, and a recent study demonstrated efficacy with co-administered saquinavir.1 Pharmacoenhancement of PIs with low dose ritonavir due to potent inhibition of CYP3A44 has been extensively reported.5–7 Improved bioavailability of all PIs has been observed with ritonavir, but to a lesser extent with nelfinavir.8 Dual PI regimens boosted with ritonavir have the potential advantage of

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maintaining both PIs within their therapeutic range, which may be of use for patients with low plasma drug concentrations below the minimum effective concentration (MEC) or as a salvage therapy. The double boosted PI regimen containing saquinavir/ritonavir/atazanavir has previously shown favourable pharmacokinetic profiles either at 1600/100/300 mg once daily doses when using saquinavir hard gel 200 mg capsules8 or at 1500/100/300 mg dose with the saquinavir 500 mg tablet formulation.10

For antiretroviral therapy to be successful, it is essential that all the drugs in a regimen attain adequate concentrations at their pharmacological site of action. Since the pharmacological target of PIs is primarily within cells infected with HIV, it is important that data on both plasma and intracellular pharmacokinetics of the drugs are obtained in order to develop a greater understanding of therapeutic failure. To date, there is no information on the intracellular levels of atazanavir, saquinavir or ritonavir when co-administered.

Multidrug-resistant efflux transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug-resistance-associated protein (MRP1), expressed on lymphocytes may provide a mechanism for reducing intracellular drug concentrations, leading to sanctuary of HIV.11,12 Previous reports demonstrate that haematopoietic peripheral blood mononuclear cells (PBMCs) express various levels of P-gp depending on the lineage13,14 and that P-gp mediates the efflux of PIs.5–17 Therefore, since P-gp has lowered PI intracellular accumulation in vitro and in vivo,18,19 considering the relationship between transporter expression on cell subsets and intracellular PI accumulation is important.

Here, we investigate the intracellular and plasma pharmacokinetics of low dose atazanavir (200 mg) plus saquinavir/ritonavir (1600/100/100 mg) once daily in HIV+ patients. The potential effect of the efflux transporters P-gp, MRP1 and BCRP on accumulation was investigated. Nine of the HIV+ patients had previously received a regimen containing saquinavir/ritonavir 1600/100 mg once daily and intracellular and plasma drug measurements were made. Therefore the effect of atazanavir on the intracellular and plasma pharmacokinetics of saquinavir and ritonavir was examined.

Methods

Materials

Lymphoprep was purchased from Nycomed Pharma AS (Oslo, Norway). CellFIX was purchased from Becton Dickinson (Oxford, UK). Mouse IgG2a negative control rPE, mouse anti-human CD4:FITC, CD8:FITC and mouse anti-human CD56:FITC were purchased from Serotec Ltd (Oxford, UK). The anti-human P-gp monoclonal antibody UIC2:rPE was obtained from Immuneotech (Marseille, France). Anti-MRP1 (QCRL-1) human (mouse) antibody and BCRP antibody (BXP-21) were purchased from Calbiochem (San Diego, CA, USA) and Abcam Ltd. (Cambridge, UK), respectively. Phosphate-buffered saline (PBS) tablets were purchased from Gibco Life Technologies Ltd (Paisley, UK). Ammonium formate, acetonitrile and methanol were purchased from Fisher Scientific (Loughborough, UK). A Hypurity SC18 column was purchased from Hypersil (Manchester, UK). Internal standard (Ro 31–9564) was a gift from Roche (Basel, Switzerland). Hanks balanced salt solution (HBSS) and all other compounds were purchased from Sigma Chemical Company Ltd (Poole, UK).

Subjects

Twelve subjects (11 male, 1 female), mean age 43.8 years (range 23–58 years), median CD4 count at screening 367 cells/mm³ (range 123–837 cells/mm³), viral load <50 copies/mL were enrolled into the study. This was a pilot intense pharmacokinetic study and 12 subjects were considered sufficient for relevant conclusions. HIV-infected patients provided written informed consent prior to participation in the study and ethics committee approval was obtained. Study participants had received a once-daily saquinavir/ritonavir (1600/100 mg) hard gel formulation regimen (Invirase®) with two nucleoside reverse transcriptase inhibitors (NRTIs) or were switched to this regimen if their saquinavir/ritonavir dosage was different. Co-administered antiretrovirals were zidovudine (n = 4), zalcitabine (n = 1), lamivudine (n = 3), didanosine (n = 3), stavudine (n = 1), abacavir (n = 4) and tenofovir (n = 5). Other concomitant drugs were atorvastatin (n = 1), testosterone (n = 2), dexamethasone spray (n = 1), megestrol (n = 1), methadone (n = 1), ketoconazole (n = 1), metformin (n = 1) and sildenafil (n = 2). Atazanavir (200 mg) once daily was added to the antiretroviral combination and pharmacokinetic analysis of atazanavir, saquinavir and ritonavir was performed at steady state 11 days after receiving atazanavir, to investigate PI exposure. This intracellular research using 200 mg of atazanavir was a sub-study of a plasma pharmacokinetic study aimed at investigating the combination of saquinavir/ritonavir with lower doses of atazanavir in order to ensure the achievement of the therapeutic atazanavir plasma concentrations while limiting the increase in bilirubin.20 Patients received a standardized meal containing 20 g of fat to improve drug absorption and intake of medication was observed. Participants provided four blood draws over a 24 h period with a maximum of 25 mL per sample withdrawn into EDTA tubes. Therefore, a total of 100 mL of blood was obtained per volunteer during the dosage interval. Blood samples were taken at 2, 6, 12 and 24 h after the morning dose and processed within 5 min of blood withdrawal.

Determination of plasma and intracellular concentrations of atazanavir, saquinavir and ritonavir in PBMCs over a 24 h dosage interval

Plasma was separated from 5 mL whole blood (centrifugation 700 g, 10 min) and PBMCs were isolated from the remaining 20 mL by density gradient centrifugation (700 g, 25 min, 4°C) at each sample time.21–23 PBMCs were washed in cold PBS and centrifuged (700 g, 6 min, 4°C). Cell density was determined by quantification on a haemocytometer following two additional washes, prior to 12 h extraction (1 mL, 60% methanol). An aliquot of PBMCs (5 × 10⁶ cells) was removed for efflux transporter analysis prior to extraction. Following extraction, PBMCs were centrifuged (2772 g, 4 min, 4°C) and the supernatant fraction transferred to a clean tube prior to evaporation to dryness. The time taken to process PBMCs from phlebotomy to methanol extraction was 1 h, ensuring that sampling conditions were ice-cold to prevent drug loss.24 For HPLC-MS/MS analysis, internal standard (Ro 31–9564; 20 μL, 100 ng/mL) was added to dried PBMC extracts (resuspended in 100 μL of distilled water). To heat-inactivated plasma samples (100 μL, 58°C, 40 min), standard curves (range: saquinavir 50–10000 ng/mL; ritonavir 100–20000 ng/mL; atazanavir 50–10000 ng/mL) and quality control samples (saquinavir 200, 1000 and 5000 ng/mL; ritonavir 400, 2000 and 10000 ng/mL; atazanavir 200, 2600 and 7350 ng/mL), internal standard (Ro 31–9564; 20 μL, 1 μg/mL) was added, prior to further extraction using diethyl ether (3 mL). The aqueous layer was frozen and the organic layer was transferred to a clean tube and evaporated to dryness. Standards,
Intracellular and plasma concentrations of atazanavir, saquinavir and ritonavir

quality control samples and plasma samples were reconstituted in mobile phase (1.5 mL) [20 mM ammonium formate buffer-acetonitrile (30:70, v/v)] and PBMC extracts resuspended in 150 µL. Each sample (100 µL) was transferred into autosampler vials prior to injection onto the column. Saquinavir, atazanavir and ritonavir were eluted on a Hypurity Elite SC(3) column (5 µm; 250 x 4.6 mm) protected by a pre-column guard (Si 60, 5 µm) with the mobile phase maintained at 1.2 mL/min. The internal standard (retention time 8.67 min) was measured using an ion trap MS/MS detection system that monitors simultaneous daughter ions (m/z 674.4/573.3, 388.2).

Saquinavir (retention time 4.58 min) was analysed by fragmentation of the parent compound and quantification of resulting fragments; monitoring of ions m/z 671.4/426.1, 268.0. Atazanavir (retention time 4.18 min) was also analysed by fragmentation of the parent compound and quantification of resulting fragments; monitoring of ions m/z 612.2, 534.2 and 335.1. Drug assays were internally validated within the Liverpool laboratory, which holds Clinical Pathology Accreditation status, and externally by the International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring in HIV Infection (KKGT, The Netherlands). The lower limit of quantification was 50 ng/mL for saquinavir and atazanavir and 100 ng/mL for ritonavir. Inter- and intra-assay variability was 9% and 6% for saquinavir, 50 ng/mL for saquinavir and atazanavir and 100 ng/mL for ritonavir.

(700 g, 5 min, 4°C). Cell pellets were subsequently resuspended, fixed (CellFIX 1:10, 1 mL; 25 min, 4°C) and transferred (100 µL) into 8 wells of a 96-well plate. Following fixing, cells were centrifuged (700 g, 5 min, 4°C) and incubated with ice-cold 90% methanol (100 µL, 10 min). HBSS (150 µL) was added to each well and centrifuged (700 g, 6 min, 4°C). Samples were washed with HBSS containing 0.01% Tween (100 µL per well) and incubated with pooled human serum (100 µL, 30 min, 22°C). Samples were centrifuged (700 g, 6 min, 4°C) and incubated with primary antibody BXP-21 (1:100 in HBSS containing 2% BSA and 0.01% Tween) or isotype control IgG2a (1:40 in HBSS containing 2% BSA and 0.01% Tween). Following antibody incubation (60 min, 22°C, 50 µL per well), samples were washed twice (150 µL HBSS containing 0.01% Tween per well) followed by centrifugation (700 g, 6 min, 4°C). Cells were then incubated with an anti-IgG2a PE-conjugated secondary antibody (50 µL per well, 1:250 in HBSS containing 0.01% Tween, 60 min, 22°C). Samples were washed twice (150 µL of HBSS containing 0.01% Tween per well) and centrifuged (700 g, 6 min, 4°C). Samples were fixed (CellFIX 1:10, 200 µL per well), transferred to flow cytometry tubes and analysed by single-colour flow cytometry.

Flow cytometry was conducted on a Coulter epics XL-MCL flow cytometer. Forward scatter (FS) and side scatter (SC) signals were presented on a linear scale dot plot and fluorescence was presented on a logarithmic scale histogram. Lymphocytes were electronically gated from the total PBMC using light-scattering properties, collecting 3000 events for each sample. Transporter expression was calculated by subtracting the median fluorescence intensity value obtained for the non-specific isotype control (IgG2a) from the median fluorescence intensity value of the specific primary antibody used to measure P-gp, MRP1 or BCRP. This is referred to as increase in fluorescence (MI).

Effect of atazanavir on the intracellular and plasma pharmacokinetics of saquinavir and ritonavir

Nine patients involved in this study receiving once-daily saquinavir/atazanavir/ritonavir 1600/200/100 mg had previously received once-daily saquinavir/ritonavir 1600/100 mg when cellular and plasma drug concentrations were determined. The studies were completed approximately 10 months apart. Therefore the effect of atazanavir on both the cellular and plasma pharmacokinetics of once-daily saquinavir and ritonavir was estimated.

Statistical analysis

Intracellular concentrations of saquinavir, atazanavir and ritonavir were calculated on the basis of a single PBMC volume of 0.4 µL and total cell density of samples. The calculated intracellular concentration of each drug is the total drug concentration associated with the cells. The areas under the concentration–time curve (AUC) for plasma and intracellular drug over the 24 h dosage interval were evaluated by non-compartmental modelling, using the linear trapezoidal rule (WinNonlin computer software). Intracellular accumulation data were quantified and presented as a ratio of the intracellular AUC to the total plasma AUC over the whole dosage interval. Elimination half-life was determined by linear regression of the 6, 12 and 24 h data points.

To verify the use of a limited number of data points for determination of AUCd-24 we used rich data from a previously published pharmacokinetic study of saquinavir/ritonavir/atazanavir 1600/100/300 mg once daily5 plus other in-house data (n = 26). Pharmacokinetic data were randomly split into development and validation sets, both containing 13 patients. Using the development
Multivariate step-wise forwards regression was used to determine predictability of using only a limited number of concentrations for determination of saquinavir or atazanavir AUC0–24. Predictive performance using the validation set of patients was evaluated using methods described previously. Mean relative prediction error (MPE%) and standard error (SE%) were used as a measure of bias and root mean squared relative prediction error (RMSE%) and standard error (SE%MSE) as a measure of precision. The %MPE or percentage deviation of the predicted AUC0–24 from the true AUC0–24 should not be significantly different from zero and the variation in predicted AUC0–24 (%RMSE) should be <15%. For saquinavir the time points of 2, 6, 12 and 24 h gave an AUC0–24 that was precise (%RMSE 9.7%) and unbiased. For atazanavir, the time points gave an AUC0–24 that was precise (%RMSE 9.7%) and unbiased. The $R^2$ values for the development and validation sets were 0.989 and 0.988 for saquinavir and atazanavir, respectively.

All data were tested for normality using the Shapiro–Wilk test. Correlations between data sets were analysed using simple linear regression analysis, since the data were normally distributed. Statistical analysis of the effect of P-gp, MRP1 or BCRP expression on lymphocyte accumulation of PIs was performed using simple linear regression analysis. Comparisons between nine patients with and without atazanavir as part of their antiretroviral regimen were analysed by Wilcoxon Rank Signed Tests. Statistical analysis was performed using Arcus Quickstat Biomedical Software Version 1.1 1997.

### Results

**Plasma and intracellular concentration of atazanavir, saquinavir and ritonavir over a 24 h dosage interval**

Saquinavir, ritonavir and atazanavir plasma and intracellular pharmacokinetic profiles are shown in Figure 1.

The main pharmacokinetic parameters illustrating saquinavir, atazanavir and ritonavir plasma and intracellular exposures, AUC0–24 coefficient of variation and accumulation ratios are illustrated in Table 1. Plasma half-lives of saquinavir, ritonavir and atazanavir (based on the 6, 12 and 24 h time points) were similar to previous reports investigating a once-daily dosage using a rich data set. A significant difference between the plasma and cellular half-life of saquinavir ($P = 0.04$) and atazanavir ($P = 0.02$) was observed, whereas the difference between ritonavir cellular and plasma half-life reached borderline significance ($P = 0.05$). No differences in atazanavir plasma and intracellular concentrations were observed between patients on tenofovir and not on tenofovir.

Total plasma and intracellular exposure (AUC0–24) of saquinavir gave a significant relationship ($r^2 = 0.42$, $P = 0.02$, 95% CI of $r$ value 0.12–0.89) and borderline significance was noted with ritonavir ($r^2 = 0.31$, $P = 0.06$). Total plasma and intracellular exposure to atazanavir showed no significant relationship ($r^2 = 0.19$, $P = 0.16$; data not shown).

Accumulation of saquinavir and ritonavir expressed as a ratio (cellular AUC0–24/plasma AUC0–24) showed a direct relationship ($r^2 = 0.91$, $P < 0.0001$, 95% CI of $r$ value 0.85–0.99). Saquinavir and atazanavir accumulation demonstrated a significant relationship ($r^2 = 0.90$, $P < 0.0001$, 95% CI of $r$ value 0.83–0.99), as did ritonavir and atazanavir accumulation ($r^2 = 0.91$, $P < 0.0001$, 95% CI of $r$ value 0.84–0.99; data not shown).

Saquinavir accumulation was significantly higher than ritonavir accumulation ($P < 0.0001$) in accordance with previous studies. Saquinavir accumulation and ritonavir accumulation were also significantly higher than atazanavir accumulation ($P < 0.0001$ and $P = 0.01$, respectively).

**Relationship between atazanavir, saquinavir and ritonavir accumulation and P-gp, MRP1 and BCRP expression on lymphocytes**

The cellular accumulation of saquinavir demonstrated no relationship to total lymphocyte cell surface expression of P-gp ($r^2 = 0.18$, $P = 0.17$, for total cells; $r^2 = 0.001$, $P = 0.95$, for CD4+ cells; $r^2 < 0.001$, $P = 0.99$, for CD8+ cells; $r^2 = 0.03$, $P = 0.57$, for CD56+ cells). Similarly, no relationship was observed between intracellular accumulation of ritonavir and P-gp expression ($r^2 = 0.11$, $P = 0.29$, for total cells; $r^2 = 0.01$, $P = 0.76$, for CD4+ cells; $r^2 = 0.01$, $P = 0.79$, for CD8+ cells; $r^2 = 0.07$, $P = 0.39$, for CD56+ cells). No relationship was observed between intracellular accumulation of atazanavir and P-gp expression ($r^2 = 0.10$, $P = 0.33$, for total cells; $r^2 = 0.01$,
Intracellular and plasma concentrations of atazanavir, saquinavir and ritonavir

Table 1. Plasma and intracellular pharmacokinetic data of saquinavir (SQV), ritonavir (RTV) and atazanavir (ATV), following administration of a once daily regimen of saquinavir/atazanavir/ritonavir 1600/200/100 mg

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Intracellular</th>
<th>Plasma</th>
<th>Intracellular</th>
<th>Plasma</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{24} (mg/L)</td>
<td>0.14 (0.04–0.91)</td>
<td>1.76 (0.02–5.18)</td>
<td>0.03 (0.01–0.1)</td>
<td>0.07 (0.02–0.47)</td>
<td>0.33 (0.1–1.8)</td>
<td>0.69 (0.03–1.8)</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>4.8 (3.9–8.3)</td>
<td>6.9 (2.2–9.1)</td>
<td>4.4 (3.2–5.4)</td>
<td>5.4 (3.6–8.7)</td>
<td>7.5 (4.6–19.5)</td>
<td>12.1 (3.3–22.3)</td>
</tr>
<tr>
<td>AUC_{0–24} (mg·h/L)</td>
<td>24.9 (13.9–323.6)</td>
<td>127 (71.5–323.6)</td>
<td>6.4 (2.3–12.4)</td>
<td>11.6 (3.6–28.6)</td>
<td>25.8 (16–65.7)</td>
<td>30.1 (13.9–71.9)</td>
</tr>
<tr>
<td>Coefficient of variance</td>
<td>56.4</td>
<td>48.7</td>
<td>47.8</td>
<td>58.7</td>
<td>47.9</td>
<td>47.0</td>
</tr>
<tr>
<td>Accumulation ratio</td>
<td>4.9 (3.4–11.0)</td>
<td>1.7 (0.9–4.6)</td>
<td>1.2 (0.7–2.6)</td>
<td></td>
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</tr>
</tbody>
</table>

Data are expressed as median (range) values (n = 12).

Table 2. Influence of atazanavir (ATV) 200 mg once daily on pharmacokinetic parameters of saquinavir (SQV) given with ritonavir (RTV) in a 1600/100 mg once-daily regimen

<table>
<thead>
<tr>
<th></th>
<th>SQV with ATV</th>
<th>SQV without ATV</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma C_{24} (mg/L)</td>
<td>0.14 (0.04–0.91)</td>
<td>0.05 (0.03–0.15)</td>
<td>0.02</td>
</tr>
<tr>
<td>Cellular C_{24} (mg/L)</td>
<td>2.03 (0.85–5.18)</td>
<td>0.61 (0.20–1.14)</td>
<td>0.02</td>
</tr>
<tr>
<td>Plasma AUC_{0–24} (mg·h/L)</td>
<td>27.0 (13.9–70.2)</td>
<td>11.5 (5.70–22.3)</td>
<td>0.004</td>
</tr>
<tr>
<td>Cellular AUC_{0–24} (mg·h/L)</td>
<td>117.2 (71.5–323.6)</td>
<td>34.9 (24.7–102.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>Plasma half-life (h)</td>
<td>4.7 (3.9–8.3)</td>
<td>4.4 (2.5–6.1)</td>
<td>0.13</td>
</tr>
<tr>
<td>Cellular half-life (h)</td>
<td>7.4 (5.5–9.1)</td>
<td>6.4 (4.0–17.7)</td>
<td>0.91</td>
</tr>
<tr>
<td>Accumulation ratio</td>
<td>4.5 (3.4–11.0)</td>
<td>3.4 (1.5–6.7)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Data are expressed as median (range) values (n = 9).

P = 0.76, for CD4+ cells; r² = 0.01, P = 0.76, for CD8+ cells; r² = 0.12, P = 0.27, for CD56+ cells.

The cellular accumulation of saquinavir demonstrated no relationship to expression of MRPI or BCRP (r² = 0.01, P = 0.76 and r² = 0.003, P = 0.87, respectively). Ritonavir accumulation demonstrated no relationship to expression of MRPI or BCRP (r² = 0.03, P = 0.62 and r² < 0.001, P = 0.99, respectively). Accumulation of atazanavir demonstrated no relationship to expression of MRPI or BCRP (r² = 0.01, P = 0.79 and r² = 0.003, P = 0.86, respectively).

Effect of atazanavir on the intracellular and plasma pharmacokinetics of saquinavir and ritonavir

The effect of atazanavir on saquinavir and ritonavir pharmacokinetic parameters is shown in Tables 2 and 3.

A significant increase in plasma saquinavir C_{24} was observed with the addition of atazanavir to the saquinavir/ritonavir regimen (median values 0.05 and 0.14 mg/L without and with atazanavir, respectively; P = 0.02). Plasma saquinavir C_{24} was not significantly changed by addition of atazanavir to the regimen (median values 0.05 and 0.03 mg/L, without and with atazanavir, respectively; P = 0.81), in agreement with previous data. No significant difference in cellular saquinavir C_{24} was observed without or with atazanavir (median values 0.17 and 0.12 mg/L, respectively; P = 0.69).

Plasma saquinavir exposure was significantly higher when atazanavir was co-administered, with an increase in AUC_{0–24} from 11.5 to 27.0 mg h/L (P = 0.004) (Table 2). Again, this is in agreement with previous studies showing atazanavir (300 mg) to significantly increase saquinavir exposure. In addition, the cellular saquinavir AUC_{0–24} was significantly increased with addition of atazanavir to the regimen from 34.9 to 117.2 mg h/L (P = 0.004). However, plasma and cellular ritonavir exposure was not significantly different with co-administration of atazanavir.

There was no significant difference in saquinavir plasma half-life (4.7 and 4.4 h, with and without atazanavir, respectively; P = 0.13) or ritonavir plasma half-life (4.4 and 4.0 h, with and without atazanavir, respectively; P = 0.65). The cellular half-life of saquinavir was not significantly different (7.4 and 6.4 h, with and without atazanavir, respectively; P = 0.91). No significant difference in ritonavir cellular half-life was observed (5.8 and 6.6 h, respectively; P = 0.55).

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Discussion

The median intracellular accumulation ratios of saquinavir, ritonavir and atazanavir indicate that they enter the intracellular compartment and accumulate within cells such that the intracellular concentration (or more strictly ‘cell-associated’ concentration) is greater than in the plasma (Table 1). The accumulation ratios of saquinavir and ritonavir reported in this study are in agreement with those in previous studies measuring accumulation ratios of co-administered saquinavir and ritonavir.\(^{19,22,23}\) However, the novel aspect of this study is the measurement of intracellular accumulation of atazanavir and the impact of atazanavir on saquinavir and ritonavir. Differential accumulation of PIs has been demonstrated within PBMCs in vivo, with saquinavir accumulation higher than ritonavir accumulation\(^{22,26}\) and in this cohort the same hierarchy of the PIs was determined (saquinavir > ritonavir > atazanavir), showing that atazanavir accumulated to the least extent. PI accumulation depends on a dynamic balance between physiochemical properties of PIs, transport by influx and efflux proteins and their affinity for plasma and cellular proteins. Since the majority of PIs are lipophilic, (including atazanavir, which has low water solubility; product information leaflet, 2004), they can penetrate the phospholipid bilayer of cellular membranes\(^{28}\) and accumulate.

A correlation was observed between plasma and intracellular saquinavir exposure (\(P = 0.02\)) and borderline significance was noted for the relationship between plasma and intracellular ritonavir exposure (\(P = 0.06\)). However, no significant relationship was observed between plasma and intracellular atazanavir exposure (\(P = 0.16\)). This suggests that the extrapolation of intracellular concentrations of saquinavir and ritonavir from simultaneous levels in plasma may be possible, in agreement with previous studies.\(^{22,29}\) However, these data and the lack of an apparent clear relationship between plasma and cellular atazanavir should not be over-interpreted, since what is crucial for drug efficacy is the free concentration inside the cell able to bind the protease enzyme. To date there is no way of determining the free concentration.

Our results concerning the three PIs studied were specific for a double PI regimen context. However, for saquinavir and ritonavir, these were consistent with previous data,\(^{22}\) where greater plasma saquinavir exposure was related to greater plasma ritonavir exposure. In addition, greater plasma atazanavir exposure was associated with higher plasma saquinavir or ritonavir exposure, providing evidence that high bioavailability of one PI in an individual is associated with a high bioavailability of another co-administered PI. The same trends were observed for intracellular exposure, so a relationship was observed between intracellular exposure of saquinavir and ritonavir, ritonavir and atazanavir and borderline significance for saquinavir and atazanavir. This suggests that the three PIs examined in this study may have a similarity in relation to the mechanism of entering or being removed from cells.

An association was observed between saquinavir and ritonavir accumulation, in agreement with previous studies;\(^{22,23}\) thus, greater intracellular saquinavir exposure was associated with greater intracellular ritonavir. In addition, a correlation was noted between saquinavir and atazanavir accumulation and between ritonavir and atazanavir accumulation. This may be related to greater systemic exposure of one PI linked to greater systemic exposure of another PI, so more PI is available in the plasma to undergo accumulation in cells.

In this examination of the data from nine patients on a saquinavir/ritonavir 1600/100 mg regimen with and without atazanavir, the saquinavir plasma \(\text{C}_{24}\) (0.14 mg/L) was above the accepted MEC (0.10 mg/L) and the median saquinavir plasma \(\text{AUC}_{0–24}\) (24.9 mg h/L) was above the target for optimal suppression\(^{30}\) when atazanavir was added to the regimen. Addition of atazanavir to a once-daily saquinavir/ritonavir regimen may be useful as an additional booster of saquinavir with ritonavir, especially in patients with borderline saquinavir concentrations about the MEC, although further efficacy studies are warranted.

Saquinavir and ritonavir demonstrated similar half-lives in cells and plasma and were within the range of previously published data.\(^{9,22,31}\) Both saquinavir and ritonavir had significantly longer intracellular half-lives than in plasma, in agreement with previous data.\(^{22}\) and atazanavir also had a longer intracellular half-life than in plasma. The median plasma half-life of atazanavir was 7.5 h, with an intracellular half-life of 12.1 h. There was no significant difference in the half-life of saquinavir or ritonavir in cells or plasma when atazanavir was co-administered. Potentially a longer intracellular half-life of saquinavir may allow greater forgiveness for missed or late doses. Thus, the intracellular penetration of PIs is clinically important, and a greater understanding of combined PI intracellular pharmacology will help our understanding of cellular resistance.

Atazanavir increased plasma and cellular saquinavir \(\text{C}_{24}\) and \(\text{AUC}_{0–24}\). However, the cellular and plasma half-lives and accumulation ratios of saquinavir and ritonavir were not significantly different when atazanavir was included in the regimen. Ritonavir and atazanavir may have independent mechanisms of boosting saquinavir and other CYP3A4 substrates. Addition of atazanavir may therefore be a future treatment option, especially for patients with very low plasma concentrations of saquinavir. In this study, six out of nine patients had a saquinavir trough concentration in plasma above the MEC after the addition of atazanavir, whereas only one patient of the nine had a saquinavir trough concentration in plasma above the MEC before the addition of atazanavir.

Intracellular drug concentrations have the potential to be influenced by multidrug resistance transporters via an efflux mechanism.\(^{11,12}\) In this study, no relationship between lymphocyte P-gp, MRPI or BCRP expression and intracellular drug accumulation of saquinavir, atazanavir or ritonavir was observed despite PIs being substrates or inhibitors of these transporters. This could be a function of the relatively small numbers of patients studied and larger studies may be needed to investigate the link between pharmacokinetics and transporter expression. However, combination of atazanavir with saquinavir/ritonavir may achieve higher drug concentrations, potentially leading to concentrations that saturate transporters. It is known that PIs are inhibitors of P-gp\(^{32,33}\) and therefore once-daily regimens with higher achieved concentrations may increase their own accumulation by reducing efflux. It is also probably much too simplistic to expect a single transporter to drive an accumulation profile, given that there is growing awareness of other transporters involved in drug efflux.

Since an atazanavir dose of 200 mg has been investigated in this study and this has been shown to lead to the achievement of lower atazanavir plasma concentrations compared with the licensed 300 mg dose,\(^{22}\) it is uncertain whether 300 mg atazanavir
Intracellular and plasma concentrations of atazanavir, saquinavir and ritonavir

will influence transmembrane transporter saturation and show different intracellular PI exposures.

In summary, this study describes the intracellular pharmacokinetics of saquinavir, atazanavir and ritonavir in patients receiving a hard gel formulation of saquinavir/ritonavir (1600/100 mg) administered once daily in combination with atazanavir (200 mg). Accumulation of each PI was unrelated to the lymphocyte surface expression of P-gp, MRP1 or BCRP. In this cohort of patients co-administration of atazanavir caused an increase in both the plasma and intracellular exposure (AUC_{0-24} and C_{24} of saquinavir, but not ritonavir. Addition of atazanavir to a once-daily saquinavir/ritonavir regimen may therefore be useful for patients with saquinavir concentrations below the MEC. However, further efficacy studies are warranted.

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Transparency declarations

None to declare.

References

3. Colonnio RJ, Thiry A, Limoli K et al. Activities of atazanavir (BMS-262832) against a large panel of human immunodeficiency virus type 1 clinical isolates resistant to one or more approved protease inhibitors. Antimicrob Agents Chemother 2003; 47: 1324–33.

