P-glycoprotein and MRP1 expression and reduced ritonavir and saquinavir accumulation in HIV-infected individuals

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Objectives: Efflux transporters may play a role in lowering intracellular drug concentrations. As the HIV protease inhibitors are substrates for the efflux transporters P-glycoprotein and MRP, we wished to investigate whether differences in expression of these transporters on human lymphocytes correlated with intracellular concentrations of ritonavir and saquinavir.

 Patients and methods: Drug efflux transporter expression (P-glycoprotein and MRP1) on peripheral blood mononuclear cells isolated from HIV-positive patients was investigated using flow cytometry. In addition, plasma and intracellular ritonavir and saquinavir concentrations were measured by HPLC/mass spectrometry. The ratio of intracellular:plasma drug concentration was used to quantify intracellular drug accumulation.

Results: Patients with lower MRP1 expression (<median) had a significantly higher accumulation of both ritonavir and saquinavir than those with higher MRP1 expression (P = 0.035, CI = –1.70 to –0.06 and P = 0.043, CI = –12.79 to –0.11, respectively). Ritonavir accumulation was significantly greater in patients with lower P-glycoprotein expression (<median) than in patients with higher expression (P = 0.014, CI = –1.56 to –0.14). There was no relationship between saquinavir accumulation in patients and P-glycoprotein expression (P = 0.219, CI = –5.02 to 2.40). Combining expression of P-glycoprotein and MRP1 (expression index, EI = [(P-glycoprotein – 1) + (MRP1 – 1) × 100]) resulted in a statistically significant relationship between transporter expression and intracellular accumulation of both saquinavir (r² = 0.195, P = 0.035) and ritonavir (r² = 0.220, P = 0.049).

Conclusion: Increased expression of P-glycoprotein and MRP1 on lymphocytes is associated with lower intracellular accumulation of saquinavir and ritonavir. These two transporters may play a role in the efflux of ronavir and saquinavir from lymphocytes in vivo.

Keywords: P-glycoprotein, MRP, accumulation, ritonavir, saquinavir

Introduction

The failure of drugs to adequately suppress HIV replication currently constitutes a major limitation to antiretroviral therapy.1 Treatment failure is multifactorial but one important cause is inadequate drug exposure as a result of poor adherence or other pharmacological factors. Low plasma drug levels are strongly related to virological failure,2 but it is ultimately the amount of free drug within cells (the site of HIV replication) that will most closely influence antiviral activity. A number of cellular and tissue compartments exist that may provide sanctuary for HIV.3,4 In these areas the virus may persist and replicate, despite apparent suppression of replication in plasma.5 It is thought that multidrug resistance transporters may play a role in lowering intracellular drug concentrations at these sites via an efflux mechanism.6,7

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P-glycoprotein and MRP (multidrug resistance associated protein) are two well characterized transporters of great interest in the field of HIV research, as their substrates include both the protease inhibitors (e.g. ritonavir and saquinavir) and the nucleoside analogues.\textsuperscript{7–12} P-glycoprotein and MRP\textsubscript{1} are known to be expressed on human lymphocytes,\textsuperscript{13,14} a major site of HIV replication and antiretroviral drug action.\textsuperscript{15}

There have been many in vitro studies illustrating the mechanisms by which these transporters may impact upon the nucleoside analogues.\textsuperscript{7} Both the protease inhibitors (e.g. ritonavir and saquinavir) and interest in the field of HIV research, as their substrates include protein) are two well characterized transporters of great differences in the expression of P-glycoprotein and MRP\textsubscript{1} on human lymphocytes would impact on intracellular protease inhibitor concentrations in an HIV-infected population.

Materials and methods

Materials

Saponin was purchased from Sigma Chemical Co. Ltd (Poole, UK). Phosphate-buffered saline (PBS) tablets were purchased from Gibco Life Technologies Ltd (Paisley, UK). Lymphoprep was purchased from Nycomed Pharma AS (Oslo, Norway) and Cellfix from Becton Dickinson (Oxford, UK). Goat anti-mouse IgG2a:RPE and mouse IgG2a negative control were purchased from Serotec Ltd (Oxford, UK). The anti-human P-glycoprotein antibody, UIC2, was obtained from Immunotech (Marseilles, France). The anti-human MRP antibody, MRP\textsubscript{m5} (specific for MRP\textsubscript{1}),\textsuperscript{19} was obtained from Kamiya Biomedical Company (Seattle, WA, USA). Ammonium formate, acetonitrile and methanol were purchased from Fisher Scientific (Loughborough, UK). Hypurity 5C18 column was purchased from Hypersil (Manchester, UK).

Patients

A total of 32 HIV-infected patients attending clinics in the Mortimer Market Centre, London, Royal Liverpool University Hospital and North Manchester General Hospital were recruited. Ethical approval for the study was obtained from relevant local ethics committees. All patients gave informed written consent. All patients at the time of study had an undetectable plasma viral load (<50 copies/mL) on combination antiretroviral therapy including a protease inhibitor. Patients undergoing treatment with drugs known to be substrates/inhibitors of P-glycoprotein or MRP (other than a protease inhibitor, such as non-steroidal anti-inflammatory drugs, anti-neoplastic agents, verapamil) were excluded from the trial. Table 1 shows patient characteristics. Venous blood samples (25 mL) were collected into lithium heparin tubes over a full dosing interval (0, 2, 4, 8, 12 h for 12 hourly dosing, and 0, 1, 2, 4, 8 h for 8 hourly dosing).

Isolation of peripheral blood mononuclear cells and drug extraction

Plasma was separated from 5 mL of blood. Peripheral blood mononuclear cells were isolated by density cushion centrifugation, adhering stringently to a protocol developed previously.\textsuperscript{20} Samples were washed in ice-cold PBS and centrifuged (700g, 6 min, 4°C). After two more washes cells were quantified using a haemocytometer before extraction in 60% methanol. Retention of PBS at 4°C and the rapidity of these wash steps are crucial for minimization of drug loss from the cells; <10% of intracellular drug effluxes out of the cells under these conditions. Following overnight extraction, samples were centrifuged (700g, 6 min, 4°C) and the supernatant fraction transferred to a glass tube prior to evaporation to dryness.

Internal standard was added to both heat-inactivated plasma (40 min, 58°C) and dried cell extracts (resuspended in 200 µL of distilled water) prior to further extraction using diethyl ether. The aqueous layer was frozen and the organic layer was transferred to a clean tube and evaporated to dryness. Samples were reconstituted in the mobile phase [10 mM ammonium formate buffer/acetonitrile (30:70, v/v)] prior to injection on the column. Protease inhibitors were eluted on a Hypurity Elite 5C18 column (5 µm particle size; 250 × 4.6 mm) with the mobile phase maintained at 1.2 mL/min. Quantification of ions resulting from fragmentation of the parent compound (protease inhibitor) was analysed using a mass spectrometer (electrospray ionization) and Xcalibur software. Saquinavir (retention time 4.2 min) and ritonavir (retention time 3.8 min) were analysed by fragmentation of the parent compounds and quantification of the resulting fragments (monitoring of ions m/z ritonavir 721.4/426.1, 296.0; saquinavir 671.4/570.3, 433.2). The internal standard (retention time 8.0 min) was monitored at m/z 674.4/573.3, 388.2.

The lower limits of detection for saquinavir and ritonavir on the column are less than 5 and 10 pg, respectively. These correspond to plasma concentrations of 375 and 750 pg/mL or peripheral blood mononuclear cell (10 × 10\textsuperscript{6} cells) concentrations of 10 and 20 ng/mL, respectively. The inter-assay coefficients of variation (CV) for saquinavir were 9.7, 3.9 and 7.1% at concentrations of 100 ng/mL, 5 µg/mL and 15 µg/mL, respectively. The intra-assay CV were 2.0, 3.5 and 6.1% at the same concentrations. The inter-assay CV for ritonavir were 8.5, 7.0 and 4.9% at concentrations of 250 ng/mL, 2 µg/mL and 8 µg/mL, respectively. The intra-assay CV were 5.5, 5.8 and 5.7% at the same concentrations.

Detection of transporter expression in human lymphocytes

Isolated human peripheral blood mononuclear cells (3.5–7.0 × 10\textsuperscript{6} cells) were fixed (Cellfix, 1.5 mL; 25 min, 25°C), then washed (1 mL PBS, 4°C; centrifugation 700g, 6 min, 4°C) and resuspended in PBS to a concentration of 2 × 10\textsuperscript{6} cells/mL.
Aliquots (200 µL) of cell suspension were transferred to 5 mL plastic sample tubes.

Three cell samples were incubated with the UIC2 antibody (2.4 mg/L) and three with the isotype control antibody IgG2a (4.8 mg/L) for 30 min on ice (unstained cell samples were used as a negative control). All cell samples were washed twice with PBS (1 mL, 4°C) followed by centrifugation (700g, 6 min, 4°C) and incubated with (R)-phycoerythrin-bound IgG2a secondary antibody (2.0 mg/L) for 30 min on ice, in the dark. The cells were washed twice with PBS (1 mL, 4°C; centrifugation 700g, 6 min, 4°C) and fixed (Cellfix, 0.5 mL). All samples were analysed via flow cytometry.

MRP1 expression was determined as above but with additional steps for permeabilization of the cells. This was necessary as the antibody MRPm5 is directed towards an internal epitope of MRP1. Isolated peripheral blood mononuclear cells were fixed and washed as described previously before resuspension in PBS containing saponin (500 mg/L) to a concentration of 2 × 10⁶ cells/mL. Samples were left for 20 min to allow permeabilization of the cells, then 200 µL of cell suspension was transferred to the individual 5 mL tubes. Cells were incubated with MRPm5 (1.2 mg/L) or isotype control IgG2a (4.8 mg/L) for 30 min on ice (unstained cells used as a negative control). Cells were then treated as described previously but all washes used PBS containing saponin.

Flow cytometry

Flow cytometry was conducted on a Coulter Epics XL-MCL flow cytometer. For each sample 5000 events were collected. Forward scatter and side scatter signals were detected on a linear scale and fluorescence was detected on a logarithmic scale.
scale. Lymphocytes were electronically gated. (R)-Phycoerythrin-positive fluorescence (P-glycoprotein expression) was followed in channel 2 (FL2) and the amount of fluorescence plotted as a histogram of FL2 staining. Data acquisition was performed using the computer program WINMDI version 2.6 to determine median FL2 fluorescence analysis values. Data were expressed as a fold increase in fluorescence (FL2), calculated by dividing the median fluorescence intensity seen with UIC2 or MRPM5 by that seen with the IgG2a isotype control for P-glycoprotein and MRP, respectively. In addition, an expression index (EI) was utilized in order to assess the combined effects of both transporters on drug efflux from cells. This was calculated as:

\[ EI = \frac{{(\text{P-glycoprotein expression} - 1) + (\text{MRP1 expression} - 1)}}{} \times 100 \]

The relationship between EI and drug accumulation was investigated using linear regression analysis.

**Statistical analysis**

Intracellular concentrations of ritonavir and saquinavir were calculated on the basis of a single peripheral blood mononuclear cell volume of 0.4 pL (determined by flow cytometry and Furman et al.21) and total cell count. The intracellular concentrations calculated were total drug associated with the cells.

Area under the curve (AUC) values for plasma and intracellular ritonavir and saquinavir were evaluated by non-compartmental modelling by using the linear trapezoid rule (TOPFIT computer software; Gustav Fischer Verlag, Stuttgart, Germany). Patients received different dosage regimens (Table 1), which made direct comparison of transporter data with either intracellular or plasma data alone unfeasible. Intracellular accumulation data were therefore quantified and presented as a ratio of intracellular AUC to the plasma AUC over a whole dosing interval. Statistical analysis of drug accumulation in peripheral blood mononuclear cells with low and high P-glycoprotein and MRP1 expression (stratified about the median) was performed using a Mann–Whitney U-test.

**Results**

The expression of P-glycoprotein (n = 32) (given as a fold increase in FL2 fluorescence with UIC2/IgG2a negative control) ranged from 1.00 to 1.87 with a median value of 1.10. The expression of MRP1 (n = 31) (given as a fold increase in FL2 fluorescence with MRPM5/IgG2a negative control) ranged from 1.00 to 2.73 with a median value of 1.22. No significant correlation was observed between CD4 count and the expression of P-glycoprotein \((r^2 = 0.001, P = 0.896; n = 32)\) and MRP1 \((r^2 = 0.023, P = 0.420; n = 31)\). No relationship was observed between the expression of the two transporters in patients \((r^2 = 0.0134, P = 0.535; n = 31)\).

The median intracellular:plasma concentration ratio of saquinavir was 2.74 (range 0.32–22.74) and ritonavir was 1.25 (range 0.20–4.19).

Stratification of the expression of P-glycoprotein and MRP1, into two groups around the median level of transporter expression, was performed for patients receiving either ritonavir (n = 23) or saquinavir (n = 18) (Figure 1). Patients with greater than median expression of MRP1 had significantly lower intracellular accumulation of both protease inhibitors studied \((P = 0.035, CI = -0.17 to -0.06\) for ritonavir, \(P = 0.043, CI = -12.79 to -0.11\) for saquinavir). Patients with a P-glycoprotein expression greater than the median showed no significant difference in saquinavir accumulation \((P = 0.219, CI = -5.02 to 2.40)\) but had lower ritonavir accumulation \((P = 0.014, CI = -1.56 to -0.14)\).

Using the parameter of EI, a statistically significant relationship was observed between expression of P-glycoprotein + MRP1 and intracellular drug accumulation in patients receiving either ritonavir (n = 23) or saquinavir (n = 18).
P-glycoprotein and MRP1 expression

Figure 2. Relationship between the ‘expression index’ [(P-glycoprotein expression – 1) + (MRP1 expression – 1)] × 100 and accumulation of (a) ritonavir (RTV; n = 23) and (b) saquinavir (SQV; n = 18). Accumulation is given as AUC\textsubscript{Intracellular}/AUC\textsubscript{Plasma}. Correlation coefficient ($r^2$) was determined by linear regression.

($r^2 = 0.195, P = 0.035; r^2 = 0.220, P = 0.049$, respectively; Figure 2).

Discussion

Sanctuary sites, which allow the ongoing replication of HIV, are potentially a major source for the generation of drug-resistant strains of HIV, which may subsequently seed back into circulation.\(^3\) Drug efflux transporters may have a role establishing and maintaining sanctuary sites by decreasing the amount of drug present within cells.\(^4\)

In vitro studies have reported decreased intracellular accumulation of ritonavir and saquinavir in cell lines over-expressing either P-glycoprotein or MRP1.\(^5\) It is unclear whether the amount of measured drug is predominantly complexed to intracellular proteins or unbound and thus able to exert its antiviral effect. Further studies are required in order to delineate this. Of equal importance, studies demonstrating that these in vitro observations are also maintained in vivo are lacking. There are no published data correlating intracellular accumulation of a drug with transporter expression in HIV-positive patients.

In this study we have demonstrated an inverse relationship between the intracellular accumulation of saquinavir or ritonavir and transporter expression in vivo. Decreased ritonavir accumulation was associated with a high expression of each transporter on lymphocytes. Decreased intracellular accumulation of saquinavir was observed with high MRP1 expression.

The combined contribution of P-glycoprotein and MRP1 to drug efflux of ritonavir and saquinavir was assessed using the EI. There are limitations to this, not least that it represents an oversimplification, since many efflux transporters have been characterized on lymphocytes and the contribution of any individual transporter to the overall efflux of drug will depend upon a number of factors including levels of expression, in vivo functional abilities and drug specificities. Nevertheless, a statistically significant relationship was observed between the EI and intracellular drug accumulation in patients receiving either saquinavir or ritonavir. Although expression of these transporters may not necessarily correlate with functional efflux, these results are the first to suggest that accumulation of the protease inhibitors in vivo may be influenced by the efflux transporters P-glycoprotein and MRP1.

In conclusion, the relationship observed between P-glycoprotein and MRP1 expression and intracellular accumulation of ritonavir and saquinavir suggests that these drug efflux transporters may have a clinically important role in limiting drug exposure within cells. These in vivo findings are in agreement with previous in vitro studies.\(^6\) Whether or not some of the biological variability in drug response may be explained by genetic polymorphisms of the MDR-1 gene,\(^22\) or other factors influencing transporter expression, e.g. pregnane X receptor, requires further investigation. The effect of agents that may induce transporter expression, including HIV drugs themselves, also requires study.

Finally, our observations suggest that specific inhibitors of these transporters could potentially modify intracellular drug accumulation. This represents one possible novel pharmacological strategy to enhance drug penetration into sanctuary sites.

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