Effect of protease inhibitor-containing regimens on lymphocyte multidrug resistance transporter expression

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Received 16 April 2003; returned 28 May 2003; revised 6 June 2003; accepted 17 June 2003

Background: Increased expression of multidrug resistance transporters, such as P-glycoprotein (P-gp), has been suggested as a potential mechanism for decreased protease inhibitor (PI) availability at certain intracellular sites and tissue compartments.

Objectives: To investigate the effect of PIs on the surface lymphocyte expression of P-gp in vitro and in vivo.

Patients and methods: Peripheral blood mononuclear cells (PBMCs) were isolated from healthy subjects (n = 15) and incubated (72 h) with 10 µM of each PI studied (saquinavir, ritonavir, nelfinavir, indinavir, amprenavir and lopinavir), or dimethyl sulphoxide (DMSO) control. PBMCs were also isolated from HIV-infected subjects (n = 50; viral load < 50 copies/mL) on a PI- or a non-PI-containing combination antiretroviral regimen. P-gp expression was analysed by flow cytometry.

Results: No differences in surface P-gp expression on lymphocytes, CD4+ or CD8+ lymphocyte subsets were observed following incubation with 10 µM saquinavir, ritonavir, indinavir, amprenavir or lopinavir in vitro. Nelfinavir, however, increased P-gp expression. In vivo, no difference in P-gp expression on total lymphocytes was observed between patients receiving a PI-containing regimen [saquinavir n = 9, ritonavir n = 6, nelfinavir n = 7, indinavir n = 7 and lopinavir/ritonavir n = 13, and two nucleoside reverse transcriptase inhibitors (NRTIs)] and patients receiving a control regimen of three NRTIs alone (n = 8).

Conclusion: This study suggests that, of the PIs, only nelfinavir increases P-gp expression in vitro, and in vivo the PI class of antiretrovirals do not increase P-gp expression on lymphocytes. It is clear that factors other than PI induction are important in the inter-individual variability in the lymphocyte expression of P-gp.

Keywords: P-glycoprotein, flow cytometry, HIV, CD4+, CD8+, HAART, multidrug resistance transporters.

Introduction

Inadequate suppression of HIV replication remains a major limitation in the achievement of treatment success.¹ ² The failure of antiretroviral therapy is a complex interplay between many factors, including poor adherence, viral resistance, and pharmacological issues such as protein binding and cellular resistance.³ ⁴

HIV continuously replicates in distinct cellular and tissue compartments, despite suppression and eradication of virus from the plasma.⁵ It has been suggested that expression of multidrug resistance transporters may provide a mechanism for reducing the intracellular drug concentrations at certain sites, providing sanctuary for HIV.⁶-⁸ P-glycoprotein (P-gp) is a well-characterized transporter, encoded by the MDR-1 gene, which mediates the cellular efflux of a broad spectrum of agents, including antiretroviral protease inhibitors (PIs).⁹-¹¹ Agents such as rifampicin and St John’s Wort have been shown to up-regulate P-gp expression in lymphocytes and in the intestine, as well as being substrates for P-gp.¹²-¹⁵ However, few studies have examined the potential up-regulatory effect of antiretroviral agents on P-gp in vivo. Molecular mechanisms responsible for the up-regulation of P-gp are believed to involve the human steroid xenobiotic receptor (SXR), a transcriptional activator of the MDR-1 and CYP3A gene, binding to an upstream regulatory region.¹²,¹⁶ Many agents that have been shown to up-regulate P-gp are also ligands for the SXR, including rifampicin and St John’s Wort.¹⁷,¹⁸ Recently, the PIs have been shown to be ligands for SXR in the hierarchy ritonav

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As other SXR ligands result in increased P-gp expression, and as SXR has been shown to be expressed in peripheral blood mononuclear cells (PBMCs) (A. Owen, S. H. Kho & D. J. Back, unpublished results), it is important to investigate if PIs up-regulate P-gp on total lymphocytes and in different cell types.

Within the immune system, lymphocytes consist of natural killer cells, B-lymphocytes and T-lymphocytes that can be further subdivided into CD4+ and CD8+ lymphocyte subpopulations. Although HIV primarily targets CD4+ lymphocytes and macrophages, there is increasing evidence that CD8+ lymphocytes are also targeted by the virus. P-gp is differentially expressed on the surface of human lymphocyte subsets, with CD8+ cells expressing more P-gp than CD4+ cells. Therefore, it is important to consider the potential effects of antiretrovirals on the expression of transporters that may influence cellular drug accumulation on specific target cells.

The aim of this study was to investigate the effect of PI treatment on P-gp transporter expression on total lymphocytes, and specific CD4+ and CD8+ lymphocyte subpopulations obtained from healthy volunteers, in vitro. In addition, we sought to determine any possible differences in surface P-gp expression on lymphocytes taken from HIV-infected subjects on combination antiretroviral therapy with plasma HIV RNA levels <50 copies/mL (26 months) recruited for the study. According to the 1993 CDC classification, five patients were category A, 26 category B, and 19 category C. Patients were receiving a regimen containing either ritonavir 600 mg twice daily (n = 6), saquinavir hard gel 600 mg three times daily (n = 9), indinavir 800 mg three times daily (n = 7), nefilnavir 1250 mg twice daily (n = 7) or lopinavir/ritonavir 400/100 mg twice daily (n = 13) with two nucleoside reverse transcriptase inhibitors (NRTIs) — either lamivudine 150 mg and zidovudine 300 mg twice daily, or stavudine 40 mg and lamivudine 150 mg twice daily. The data were compared with a control group of patients receiving a regimen containing only three NRTIs (zidovudine 300 mg, abacavir 300 mg, lamivudine 150 mg twice daily; n = 8) with no previous exposure to a PI-containing regimen. CD4 counts in each of the patient groups (receiving a PI-containing regimen or an NRTI control) were similar (range 423–515 cells/mm³).

Materials and methods

Materials

Lymphoprep was purchased from Nycomed Pharma AS (Oslo, Norway). CellFIX was purchased from Becton Dickinson (Oxford, UK). Dimethyl sulfoxide (DMSO) and Hank’s balanced salt solution (HBSS) were obtained from Sigma Chemical Co. Ltd. (Poole, UK). Mouse IgG2a negative control:rPE, goat anti-mouse IgG2a:rPE, mouse anti-human CD4:FITC, mouse anti-human CD8:FITC and mouse IgG2a negative control were purchased from Serotec Ltd (Oxford, UK). The anti-human P-gp monoclonal antibodies UIC2 and UIC2:PE were obtained from Immunotech (Marseille, France). Phosphate buffered saline (PBS) tablets were purchased from Gibco Life Technologies Ltd. (Paisley, UK). Ritonavir and lopinavir were kindly donated by Abbott Laboratories (Chicago, USA), indinavir by MSD (New Jersey, USA), nelfinavir by Agouron (San Diego, USA), saquinavir by Roche (Basel, Switzerland) and amprenavir by GlaxoSmithKline (Harlow, UK).

The effect of PI incubation on P-gp expression on total lymphocytes, CD4+ and CD8+ cells in vitro

The in vitro effect of PI incubation on P-gp expression on total lymphocytes, CD4+ and CD8+ subpopulations was assessed in blood samples obtained from 15 healthy volunteers (nine males and six females, aged 21–52 years). The local ethics committee approved the study, and informed consent was obtained from volunteers. Blood samples (60 mL) were obtained and PBMCs isolated by density gradient centrifugation. PBMCs (>40 × 10⁶ cells) were resuspended in HBSS to a density of 5 × 10⁶ cells/mL, and transferred into eight separate wells. One well was left untreated (negative control), and 5 µL of DMSO (drug vehicle control), saquinavir, ritonavir, nelfinavir, indinavir, amprenavir or lopinavir was added to the other wells, respectively, producing a final drug concentration of 10 µM for each PI. Following incubation (72 h, 37°C), the contents of each well were transferred into separate 5 mL tubes. All samples were washed twice (1 mL HBSS, 4°C; centrifugation 700g, 6 min) and the cells fixed by resuspension in CellFIX (1:10, 1 mL, 25 min, 4°C). Cells were then centrifuged (700g, 6 min, 4°C) and resuspended in HBSS (2 mL). Aliquots (200 µL) of each cell suspension were transferred into clean 5 mL tubes for incubation with different combinations of antibodies. Four cell samples were incubated with either CD4 or CD8:FITC (2.5 µg/mL) primary conjugated antibodies, and two samples from each subpopulation were counterstained with UIC2:rPE (375 ng/mL), or with isotypically matched controls (IgG2a: 2.5 µg/mL). Unstained cell samples were used as a negative control (two tubes). Following antibody incubation (10 µL, 90 min, 4°C), all samples were washed twice (1 mL HBSS, 4°C) followed by centrifugation (700g, 6 min, 4°C). Samples were then fixed (CellFIX 1:10, 0.5 mL), and P-gp expression determined by dual colour flow cytometry on total lymphocytes, CD4+ and CD8+ cell subsets.

Subjects

HIV-infected patients (n = 50; 45 males and five females), mean age 41 (range 25–64 years), median CD4 count 467 (range 179–850) cells/mm³, on antiretroviral therapy with plasma HIV RNA levels <50 copies/mL (26 months) were recruited for the study. According to the 1993 CDC classification, five patients were category A, 26 category B, and 19 category C. Patients were receiving a regimen containing either ritonavir 600 mg twice daily (n = 6), saquinavir hard gel 600 mg three times daily (n = 9), indinavir 800 mg three times daily (n = 7), nefilnavir 1250 mg twice daily (n = 7) or lopinavir/ritonavir 400/100 mg twice daily (n = 13) with two nucleoside reverse transcriptase inhibitors (NRTIs) — either lamivudine 150 mg and zidovudine 300 mg twice daily, or stavudine 40 mg and lamivudine 150 mg twice daily; n = 8) with no previous exposure to a PI-containing regimen. CD4 counts in each of the patient groups (receiving a PI-containing regimen or an NRTI control) were similar (range 423–515 cells/mm³).

The effect of PI-containing regimens on P-gp expression on lymphocytes in vivo

Blood samples (25 mL) were obtained from HIV-positive patients, and PBMCs isolated, as previously described. The local ethics committee approved the study, and informed consent was obtained from patients. P-gp expression was measured on total lymphocytes as opposed to cell subsets, because of low cell densities attained from HIV-positive patients. Isolated PBMCs (3.5–7.0 × 10⁶ cells) were fixed (CellFIX 1:10, 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 × 10⁶ cells/mL. Aliquots (200 µL) of cell suspension were transferred to 5 mL plastic sample tubes. Three cell samples were incubated (30 min, 4°C) with UIC2 antibody (2.4 µg/mL) and three with the isotype control antibody, IgG2a (4.8 µg/mL). Unstained cell samples were used as a negative control. All samples were washed twice (1 mL PBS, 4°C), followed by centrifugation (700g, 6 min, 4°C) and incubation (30 min, 4°C) with R-phycocerythrin bound-IgG2a secondary antibody (2.0 µg/mL). The cells were washed twice (1 mL PBS, 4°C), followed by centrifugation (700g, 6 min, 4°C) and resuspended in CellFIX (1:10, 0.5 mL). P-gp expression was determined by single colour flow cytometry using a previously published method.

Flow cytometry

Flow cytometry was conducted on a Coulter Epics XL-MCL flow cytometer. Forward scatter and side scatter signals were detected on a linear scale dot plot, and fluorescence was detected on a logarithmic scale histogram. Lymphocytes were electronically gated from the total PBMC population, using light scattering properties, collecting 5000 events for each sample. P-gp expression on subsets of lymphocytes was performed as described previously. Briefly, this involves separating cells into the
particular subset of interest (either CD4+ or CD8+) by specific antibodies conjugated to an FITC fluorochrome that fluoresces in channel 1 (FL1). A flow cytometric dot plot of FL1 versus FL2 (channel 2) allows the cell subset population to be isolated from the total lymphocyte population. The specific P-gp antibody (UIC2) is detected in channel 2 (FL2), thus P-gp expression can be assessed on the cell surface of a particular population. P-gp expression on lymphocytes and subsets was calculated by subtracting the median fluorescence intensity value obtained for the non-specific isotype control from the median fluorescence of the P-gp specific antibody, referred to as median increase (MI) in fluorescence.

**Statistical analysis**

Data are expressed as MI in fluorescence ± S.D. unless stated otherwise. Statistical analyses of P-gp expression on lymphocytes and subsets in vitro and on lymphocytes in vivo were performed using the non-parametric Kruskal–Wallis analysis of variance test (Arcus Quickstat Biomedical Software Version 1.1; 1997).

**Results**

**In vitro PI incubation**

In comparison with the DMSO vehicle control, neither saquinavir, ritonavir, indinavir, lopinavir nor amprenavir caused a statistically significant change in P-gp expression on total lymphocytes, CD4+ or CD8+ lymphocyte subpopulations in vitro (Figure 1a, b and c, respectively). However, nelfinavir, in relation to DMSO, produced a statistically significant median increase in P-gp expression on total lymphocytes (MI values nelfinavir 0.323 and DMSO 0.212; P = 0.033), CD4+ cells (MI values nelfinavir 0.225 and DMSO 0.123; P = 0.023) and CD8+ cells (MI values nelfinavir 0.443 and DMSO 0.317; P = 0.030). Individual nelfinavir data are shown in Figure 2. There was a trend with ritonavir incubation in total lymphocytes and CD8+ subsets to increased P-gp expression, although this was not statistically significant (P = 0.066 and P = 0.058, respectively). To assess intra-subject variability on the effect of nelfinavir on P-gp expression on total lymphocytes, CD4+ and CD8+ subsets, blood samples were obtained from one healthy subject on seven different occasions. Nelfinavir in relation to DMSO significantly increased P-gp expression on total lymphocytes (MI values nelfinavir 0.323 and DMSO 0.207; P < 0.0001), CD4+ cells (MI values nelfinavir 0.202 and DMSO 0.113; P = 0.023) and CD8+ cells (MI values nelfinavir 0.423 and DMSO 0.200; P = 0.049). The intra-individual mean coefficients of variation for P-gp on total lymphocytes, CD4+ and CD8+ subpopulations were 18.8%, 38.9% and 52.5%, respectively.

**Effect of PI-containing regimens in vivo**

P-gp expression was similar on lymphocytes across the various PI-treated groups in the HIV-infected patient cohort. Figure 3 illustrates the results observed from flow cytometric analysis of P-gp expression from patients receiving PI- and non-PI-containing regimens.

**Discussion**

This study illustrates that of the PIs only nelfinavir increased P-gp expression on lymphocytes, CD4+ and CD8+ lymphocyte subsets in vitro. Previously, the PIs saquinavir, ritonavir, nelfinavir, indinavir and amprenavir have been implicated in increasing P-gp expression in a vinblastine-selected colonic adenocarcinoma cell line with the hierarchy ritonavir>nelfinavir>amprenavir>saquinavir>indinavir. This finding was later supported by another study, which demonstrated increased intestinal P-gp expression in both amprenavir- and nelfinavir-treated rats, and in an intestinal human cell line. More recently, however, it has been demonstrated that after PI incubation (10 µM) with PBMCs in vitro, only nelfinavir statistically increased P-gp expression. This study demonstrated cell viability via an MTT formazan formation cytotoxicity assay, illustrating that 72 h incubation of cells with PIs at 10 µM exhibited no alteration in MTT turnover, hence no toxicity was observed. At higher concentrations, all PIs increased P-gp expression, but this was secondary to toxicity and implied up-regulation as a stress response. Drugs such as rifampicin have been shown previously to be ligands for SXR, and may utilize this mechanism to increase P-gp expression. However in our study,
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The inter-individual effect of nelfinavir (NFV) in comparison with drug vehicle control (DMSO) on P-gp expression on total lymphocytes, CD4+ and CD8+ subsets of lymphocytes in vitro. Individual data are shown (n=15), with each point representing a single experiment in a single healthy subject; median expression value is indicated by the bar *P<0.05.

Figure 2.

The effects of PI-containing regimens on P-gp lymphocyte expression in vivo. HIV-infected patients were treated with two NRTIs, and saquinavir (SQV n = 9), ritonavir (RTV n = 6), nelfinavir (NFV n = 7), indinavir (IDV n = 7) and lopinavir/ritonavir (LPV/r n = 13). Comparisons were also made with HIV patients treated with a triple NRTI regimen (NRTI n = 8). Data are expressed as MI in fluorescence ± S.D. and analysed by Kruskal–Wallis multiple comparison test.

Figure 3.

The PI effects did not lead to an increase in P-gp expression in vivo, despite being ligands for SXR, suggesting that the in vitro increase in P-gp expression caused by nelfinavir may have little clinical relevance. Therefore, factors other than SXR may also be important in the up-regulation of P-gp, for example other nuclear receptors, or recruitment of co-regulators may be involved in enhanced transcription of MDR-1.18,20

There is a large inter-individual variability in the expression of P-gp, CYP3A427 and SXR,18 all of which are important in drug disposition. This is a likely explanation for the wide variability in plasma concentrations of PIs observed in clinical practice, as the PIs are substrates for P-gp,10 CYP3A427 and SXR,18 all of which may affect plasma drug clearance. As large inter-individual variability exists in transporter expression, factors other than PI induction are likely to be responsible. These include genetic variability with genes that encode MDR-130 or PXR.31 The increase in P-gp on total lymphocytes in one healthy volunteer analysed on seven separate occasions was highly significant in comparison with simultaneous CD4+ and CD8+ levels. This may mean that within this individual the CD56+ cells contributed a high percentage of the total lymphocytes, and P-gp was increased leading to a higher overall increase in P-gp on total lymphocytes. This demonstrates that assays that measure P-gp expression on subsets of lymphocytes may provide additional information.

In accordance with the in vitro data (with the exception of nelfinavir), our in vivo data show no increase in P-gp expression on lymphocytes from patients on PI-containing regimens, in comparison with PI-sparing regimens. Although few studies have investigated this phenomenon, our data are in agreement with previous studies, which observed no change in P-gp expression on peripheral CD4+ blood cells from patients on PI therapy,32 and a lack of induction of human lymphocyte P-gp by highly active antiretroviral therapy (HAART).33

Our in vitro data focus on the effect of PIs on P-gp expression on CD4+ and CD8+ lymphocyte subsets. A differential P-gp expression in the subsets was detected, CD8>CD4, a finding in agreement with previous studies.19,20,22 Although no direct comparisons can be made between this in vitro model and the in vivo data, nelfinavir increased P-gp expression by a similar magnitude on total lymphocytes and both CD4+ and CD8+ cell subsets in vitro, but not in vivo. This suggests that the lack of effect of nelfinavir observed in vivo might not be due to altered CD4+ and CD8+ ratios in patients. One of many factors that may be responsible for this nelfinavir discordance in vitro and in vivo is SXR, which also targets regulatory sequences in genes encoding cytochrome enzymes such as CYP3A4,13,34 an important enzyme in the metabolism of PIs.29 Therefore, if any PI causes increased expression of cytochrome enzymes (e.g. CYP3A4) in vivo, it may lead to an enhanced metabolism of a drug, and therefore lower circulating plasma concentrations of that drug. Large variability in the free plasma concentration of PIs exist in patients for a variety of reasons, including concurrent therapy and plasma protein binding. These factors could also alter the concentration of PI available for P-gp induction in vivo.

In summary, this study illustrates that P-gp does not appear to be increased by the PIs, either in vitro or in vivo. P-gp clearly plays a role in drug disposition, reducing oral bioavailability of drugs in the gut and liver, and contributing to tissue and cell sanctuary sites in the brain, genital tract and lymphocytes. Theoretically, by up-regulating the expression of an efflux transporter, the PIs may decrease their own efficacy by lowering effective intracellular concentrations. Therefore, the lack of any induction in P-gp expression observed in this study is of pharmacological benefit in terms of antiretroviral therapeutic success.

Acknowledgements

The authors gratefully acknowledge the assistance of Diana Aldam and David Cornforth (Royal Free and University College Medical School). This study was funded in part by a grant from the British Society for Antimicrobial Chemotherapy (BSAC). Dr P. Hoggard
was funded by BSAC and J. Ford by AVERT. Some of these results have been presented previously as an oral presentation at the Fourth International Workshop on Clinical Pharmacology of HIV Therapy; 27–29 March 2003, Cannes, France.

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