Intracellular accumulation of efavirenz and nevirapine is independent of P-glycoprotein activity in cultured CD4 T cells and primary human lymphocytes

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Background: Interaction of antiretrovirals with drug transporters such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), breast cancer resistance protein (BCRP) and solute carrier organic anion transporter (SLCO) may influence the emergence of viral mutants by altering intracellular drug concentrations. Here we characterize the effect of transporter expression in a variety of cell types such as control CEM, CEMVBL (P-gp-overexpressing), CEME1000 (MRP1-overexpressing), MT4, control MDCKII, MDCKIIMDR1 (P-gp-overexpressing) and peripheral blood mononuclear cells (PBMCs) on the uptake of [14C]efavirenz and [3H]nevirapine. We also investigated the lipophilicity of [14C]efavirenz and [3H]nevirapine.

Methods: The expression of P-gp, MRP1, MRP2, SLCO1A2, 1B1, 1B3, 2B1, 3A1 and 4A1 was assessed by PCR. Inhibitors of P-gp (XR9576, GF120918, dipyridamole) and MRP (MK571, frusemide, dipyridamole), and SLCO substrate or inhibitor (estrone-3-sulphate or montelukast, respectively) were used to study the role of drug transporters in the accumulation of [14C]efavirenz and [3H]nevirapine. Lipophilicity was measured by the octanol/saline partition coefficient.

Results: CEM cells, MT4 cells and PBMCs express various SLCO isoforms, with SLCO3A1 detected in all of the cells. XR9576, dipyridamole and GF120918 had no effects on the accumulation of [14C]efavirenz, while MK571 and frusemide produced variable effects in the cells. The accumulation of [14C]efavirenz was significantly decreased in all the cells by montelukast and estrone-3-sulphate.

Conclusions: P-gp expression had no effect on the accumulation of [14C]efavirenz and [3H]nevirapine. MRP1/2 expression, lipophilicity and SLCO-like transporters (possibly SLCO3A1) may have greater influence on the accumulation of [14C]efavirenz than [3H]nevirapine.

Keywords: transport, MRP, SLCO/OATP

Introduction

Drug transporters act as a barrier to drug absorption, distribution, metabolism and excretion. Drug efflux pumps including P-glycoprotein (P-gp) and multidrug resistance-associated proteins 1 and 2 (MRP1, MRP2), influx pumps such as organic anion transporting polypeptides/solute carrier organic anion transporters (OATPs/SLCOs) and solute carrier (SLC) transport proteins transport antiretrovirals from several cellular compartments.1–3 Clearly drug efflux by transporters expressed at sites such as the blood–brain and blood–testis barriers and lymphocytes may decrease drug penetration into these organs, limiting the efficacy of substrate drugs and thereby allowing the evolution of viral mutants.

Given the potential relevance of drug transporters to the disposition of antiretrovirals, an increased understanding of how transporters may impact viral suppression is essential for optimizing therapy. Data on the interaction of the non-nucleoside reverse transcriptase inhibitors such as efavirenz and nevirapine with drug transporter proteins are conflicting.4–9

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The central focus of the current study is to investigate the expression and role of drug efflux transporters (e.g. P-gp and MRP1s) and influx transporters (SLCOs) on the accumulation of [14C]efavirenz and [3H]nevirapine in cell lines and isolated primary lymphocytes.

Materials and methods

Reagents and chemicals
RPMI 1640 medium, Hank’s balanced salt solution, frusenide, dipyridamole and lymphoprep were obtained from Sigma Chemical Co. (Poole, UK). XR9576, MK571, GF120918, montelukast and frusenide were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). Other cell types used in this study were MT4 (AIDS Reagent Project, NIBSC, UK) and A549 (ECACC 86012804; used as a positive control for MRP2 expression). MDCKIICTRL and MDCKIIABCB1 (ABCB1) were gifts from Professor P. Borst (The Netherlands Cancer Institute, Amsterdam). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

[14C]Efavirenz (specific activity, 266 μCi/mm) was a gift from Bristol-Myers Squibb; [3H]nevirapine (specific activity, 1.6 Ci/mm) and [14C]mannitol (specific activity, 50 mCi/mm) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA); [3H]saquinavir (specific activity, 26 μCi/mg) was donated by Roche Pharmaceuticals (Welwyn Garden City, UK). PCR reagents were sourced as described previously.3

Isolation of peripheral blood mononuclear cells (PBMCs)

Blood buffy coat samples from healthy volunteers (n=5) were obtained from the blood transfusion service (Manchester, UK) from which PBMCs were isolated using lymphoprep following the manufacturer’s instructions.

Ethics

No ethical approval was required in the collection and use of the blood products from the blood transfusion services.

Isolation of RNA

Total RNA was extracted from the cells (1×10⁷; PBMCs, CEM, CEMVBL, CEME1000, MT4 and A549) and reverse transcribed to cDNA as described previously.3

PCR analyses

Expression of mRNA for MDR1, MRPI, MRPII and SLCOs was assessed by real-time RT–PCR using primers and probes obtained through the Applied Biosystems (New Jersey, USA) Assays-on-Demand range (http://www.appliedbiosystems.com). PCR primer sequences, incubation conditions and analysis were as described previously.3

Octanol/saline partition coefficient

The lipophilicity of [14C]efavirenz and [3H]nevirapine was measured as described previously.3

Transport of [14C]efavirenz and [3H]nevirapine and the effects of specific inhibitors on transport

The accumulation of [14C]efavirenz and [3H]nevirapine was measured by incubating the cells (5×10⁶ cells/mL for cell lines and 10×10⁶ cells/mL for PBMCs) in the absence and presence of fixed concentrations of inhibitors: XR9576 (P-gp inhibitor; 0.01–1 μM), MK571 (MRP inhibitor; 1–100 μM), GF120918 (P-gp inhibitor; 50 μM), dipyridamole (P-gp/MRP1 inhibitor; 50 μM) and influx inhibitors montelukast (10–100 μM) and estrone-3-sulphate (1–100 μM) as described previously.1

Trans epithelial transport of [3H]nevirapine across MDCKII cells

In another experiment, apically and basolaterally directed trans epithelial transport of [3H]nevirapine and [3H]saquinavir (positive control) across MDCKCTRL and MDCKIIABCB1 cells was assessed in the absence and presence of XR9576 (1 μM) as previously described.10

Statistical analysis

Data are expressed as means ± SD. The Shapiro–Wilk test was used to assess the distribution of the data. Statistically significant differences between controls and test samples were then assessed by paired t-test. Significance between controls and test samples was assumed if P<0.05.

Results

Expression of drug transporters

We have previously shown that CEM cells, CEMVBL cells, CEME1000 cells and PBMCs express different OATP/SLCO transcripts.3 MT4 cells showed transcripts of SLCO2B1, SLCO3A1 and SLCO4A1, as well as MRPI and MRPII. As expected, MRPI and MRPII transcripts were detected in CEME1000 and CEMVBL cells; however, no MDR1 transcript was detected in MT4 cells (data not shown).

Octanol/saline partition coefficient

The log D values for [14C]mannitol (control), [14C]efavirenz and [3H]nevirapine were –2.7, 5.1 and 1.2, respectively.

Transport of [14C]efavirenz and [3H]nevirapine and the effects of inhibitors of drug efflux/influx transporters on accumulation

There was no differential accumulation of [14C]efavirenz in CEM cells (CEM, 124.3±35.6; CEMVBL, 101.9±23.6; and CEME1000: 132.4±15.3; Figure 1a). MK571 (50 μM) had no effect on the accumulation of [14C]efavirenz in the CEM cells (Figure 1b). Addition of montelukast (50 μM) and estrone-3-sulphate (100 μM) reduced the cellular accumulation ratio (CAR) of [14C]efavirenz in the cells from 115.6±10.2 to 76.3±4.2 (P=0.007) and 75.4±7.2 (P=0.009), respectively (Figure 1b).

The different PBMCs had a variable baseline CAR of [14C]efavirenz (135.1±91.8). Inhibitors of drug efflux...
transporters such as MK571, XR9576, frusemide, dipyridamole, and GF120918 exhibited variable effects. XR9576, dipyridamole, and GF120918 did not alter the accumulation of [14C]efavirenz in the PBMCs. Unexpectedly MK571 significantly \((P = 0.04)\) reduced the CAR of [14C]efavirenz, while frusemide significantly \((P = 0.05)\) increased the accumulation of [14C]efavirenz. In contrast, estrone-3-sulphate and montelukast significantly decreased the CAR of [14C]efavirenz \((P = 0.01\) and \(P = 0.04\), respectively; Figure 1c).

The baseline CAR of [14C]efavirenz in MT4 cells was not significantly altered by XR9576 (87.5 ± 1.2 versus 83.6 ± 3.8) or MK571 (87.5 ± 1.2 versus 84.3 ± 6.4). However, the CAR of [14C]efavirenz was significantly reduced by montelukast \((37.8 ± 7.2, P < 0.001)\) and estrone-3-sulphate \((70.2 ± 6.0, P < 0.01\), Figure 1d). MK571, XR9576 and estrone-3-sulphate did not alter the accumulation of [3H]nevirapine in CEM and MT4 cells (Figure 2a–d). However, while montelukast did not alter the transport of [3H]nevirapine in the MT4, CEM and CEMVBL cells (Figure 2d and e), it significantly \((P = 0.02)\) decreased accumulation in CEME1000 cells (Figure 2e). None of the inhibitors of drug transporters altered the accumulation of [3H]nevirapine in the PBMCs tested (Figure 2f).

**Apical and basolateral transport of [3H]nevirapine is independent of P-gp**

We observed no differential apical or basal transport of [3H]nevirapine in the MDCKII\_CTL and MDCKII\_MDR1 cells, with XR9576 (at 1 \(\mu M\)) having no effect on transport (Figure 2g). In a similar experiment, using [3H]saquinavir as a control, we observed that inhibition of P-gp with XR9576 (1 \(\mu M\)) significantly altered the transepithelial transport of [3H]saquinavir (data not shown).

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**Figure 1.** CAR of EFV and the effects of inhibitors of drug transporters. (a) CAR of EFV in CEM, CEMVBL and CEME1000 cells. (b) Effects of 50 \(\mu M\) MK571, 100 \(\mu M\) E-3-S and 50 \(\mu M\) montelukast on the CAR of EFV in CEM cells. (c) Effects of inhibitors of drug efflux (50 \(\mu M\) MK571, 1 \(\mu M\) XR9576, 50 \(\mu M\) frusemide, 50 \(\mu M\) dipyridamole and 50 \(\mu M\) GF120918) and influx (100 \(\mu M\) E-3-S and 50 \(\mu M\) montelukast) transporters on the accumulation of EFV in PBMCs \((n = 5, \text{with each PBMC sample assayed in quadruplicate})\). (d) Effects of 50 \(\mu M\) MK571, 1 \(\mu M\) XR9576, E-3-S and 50 \(\mu M\) montelukast on the CAR of EFV in MT4 cells. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.0001\) (compared with non-treated controls by paired \(t\)-test). Results are expressed as mean CAR ± SD \((n = 4)\). EFV, efavirenz; E-3-S, estrone-3-sulphate.
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Figure 2. Effects of inhibitors of drug transporters on the accumulation of NVP. (a) MK571 (0-100 µM), (b) XR9576 (0-1 µM), (c) E-3-S (0-100 µM) in CEM and its variant cells. (d) E-3-S and montelukast (0-100 µM) in MT4 cells. (e) Montelukast (0-100 µM) in CEM and its variant cells. (f) Effects of 50 µM MK571, 1 µM XR9576, 50 µM frusemide, 50 µM dipyridamole, 50 µM GF120918, 100 µM E-3-S and 50 µM montelukast in PBMCs (n=5, with each PBMC sample assayed in quadruplicate). Apically directed (filled circles and broken lines) and basolaterally directed (open squares and continuous lines) transepithelial transport of [3H]NVP across MDCKII-CTL and MDCKII-MDR1 cells in the absence and presence of 1 µM XR9576. Transport was measured as the percentage of the total drug detected in the receiver compartment of the transwell per unit time. Data points represent mean ± SD (n=4). *P<0.05, compared with non-treated controls. NVP, nevirapine; E-3-S, estrone-3-sulphate.
Discussion

The ability of antiretroviral agents to target infected cells may be influenced by the chemistry of the drug, drug-metabolizing enzymes, drug transporters and other factors.

Here, we screened CEM cells, MT4 cells and PBMCs for expression of SLCO1A2, 1B1, 2B1, 3A1, 4A1 and 1B3 transcripts by RT–PCR and obtained the same results as those previously published.3 MT4 cells expressed SLCO2B1, SLCO3A1 and 4A1. Both the CEM and MT4 cell line models differed from PBMCs in their expression profiles; the latter were found to express only SLCO3A1.3,11 A surprising finding in the present study was the lack of transcript for MDR1 in MT4 cells. This cell line is frequently used to assess antiviral activity via virally induced cytopathic effects, demonstrating that to be able to formulate a robust explanation of the mechanistic basis of the intracellular accumulation and activity of substrate drugs one needs an adequate understanding of the transporter profiles of the cells being used.

$[^{14}\text{C}]$Efavirenz was more lipophilic than $[^{3}\text{H}]$nevirapine (log $D = 5.1$ versus 1.2, respectively). The CAR of $[^{14}\text{C}]$efavirenz was ~55, 25 and 45 times more than that of $[^{3}\text{H}]$nevirapine in CEMs, MT4 and PBMCs (Figures 1 and 2). Our measured log $D$ values for efavirenz are in agreement with previous studies (5.412 versus 5.1), but that for nevirapine was not (2.0513 versus 1.2). This is probably because the value reported by Kasim et al.13 was based on an estimated/calculated value rather than an experimental value.

We found no evidence that P-gp and MRP1 reduce the accumulation of $[^{14}\text{C}]$efavirenz in cultured cells. However, frusemide (MRP1/2 inhibitor) significantly ($P < 0.05$) increased the accumulation of $[^{14}\text{C}]$efavirenz in the PBMCs (Figure 1c), suggesting some evidence that it may be transported by MRP1 or MRP2. Nevertheless, the MRP inhibitor, MK571 caused an unexpected and borderline significant ($P < 0.05$) reduction in the accumulation of $[^{14}\text{C}]$efavirenz in the PBMCs (Figure 1c), suggesting that MK571 may inhibit drug influx transporters or compete with efavirenz for influx.

Previous studies in Caco-2 cells, expressing a variety of transport proteins, and in GPNt cells provided some evidence that $[^{14}\text{C}]$efavirenz is not a P-gp substrate.2,7 Additionally, we observed no correlation between P-gp expression and the intracellular concentration of efavirenz in PBMCs from HIV-infected subjects,14 suggesting that the cellular accumulation of efavirenz may not be affected by P-gp or that multiple factors rather than a single protein control the entry and retention of a drug. However, prolonged exposure of LS180 V cells to efavirenz induced P-gp expression,7 a finding supported in PBMCs by work from our laboratory.4

The CAR of $[^{14}\text{C}]$efavirenz was decreased in each of the cell types by incubation with the OATP substrate and inhibitor estrone-3-sulphate, and montelukast, a known inhibitor of OATPs,3,15 suggesting that an SLCO-like uptake transporter (possibly SLCO3A1) may be responsible for the accumulation of $[^{14}\text{C}]$efavirenz in these cells.

Our data suggest that $[^{3}\text{H}]$nevirapine is not a substrate of P-gp and MRP1/2, and this stems from three important direct observations: (i) there was no differential accumulation of $[^{3}\text{H}]$nevirapine in the CEM cells; (ii) relatively specific inhibitors of MRP, P-gp and OATP did not alter the accumulation of $[^{3}\text{H}]$nevirapine in any of the cell types tested; and (iii) there was
no differential apical or basal transport of $[^3]H$nevirapine in the MDCKIICTL and MDCKII{	extsubscript{MDR1}} cells. Our direct inhibition studies provide strong evidence for the lack of involvement of P-gp in the accumulation of $[^3]H$nevirapine, and this is in agreement with previous observations. Why are these data different from the clinical data generated in our unit which showed an association between the intracellular exposure of $[^3]H$nevirapine and P-gp expression in HIV-infected individuals, but not with efavirenz intracellular concentrations? Direct inhibition of transporter activity suggests differential transporter involvement and lack of a class effect in the accumulation of $[^3]H$nevirapine. 

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