Differential Modulation of P-Glycoprotein Expression and Activity by Non-Nucleoside HIV-1 Reverse Transcriptase Inhibitors in Cell Culture

Elke Störmer,[1] Lisa L. von Moltke,[1] Michael D. Perloff,[1] and David J. Greenblatt[1,2]

Received January 24, 2002; accepted March 28, 2002

Purpose. This study investigated the effects of the non-nucleoside HIV-1 reverse transcriptase inhibitors (NNRTI) nevirapine (NVR), efavirenz (EFV), and delavirdine (DLV) on P-glycoprotein (P-gp) activity and expression to anticipate P-gp related drug-drug interactions associated with combination therapy.

Methods. NNRTIs were evaluated as P-gp substrates by measuring differential transport across Caco-2 cell monolayers. Inhibition of P-gp mediated rhodamine123 (Rh123) transport in Caco-2 cells was used to assess P-gp inhibition by NNRTIs. Induction of P-gp expression and activity in LS180V cells following 3-day exposure to NNRTIs was measured by western blot analysis and cellular Rh123 uptake, respectively.

Results. The NNRTIs showed no differential transport between the basolateral to apical and apical to basolateral direction. NNRTI transport in either direction was not affected by the P-gp inhibitor verapamil. DLV inhibited Rh123 transport, causing a reduction to 15% of control at 100 μM (IC_{50} = 30 μM). NVR caused a concentration-dependent induction of P-gp expression in LS180V cells resulting in a 3.5-fold increase in immunoreactive P-gp at 100 μM NVR. Induction attributable to EFV and DLV was quantitatively smaller. NVR significantly reduced cellular uptake of Rh123 into LS180V cells, indicating increased drug efflux due to induced P-gp activity; effects of EFV and DLV were smaller. Acute DLV treatment of LS180V cells previously induced with NVR or ritonavir did not reverse the decreased Rh123 cell accumulation.

Conclusions. NNRTIs show differential effects on P-gp activity and expression *in vitro.* Clinical studies are required to elucidate the clinical importance of potential drug interactions.

KEY WORDS: P-glycoprotein; induction; inhibition; non-nucleoside reverse transcriptase inhibitors; Caco-2; LS180.

INTRODUCTION

Non-nucleoside inhibitors of the HIV-1 reverse transcriptase have gained increasing importance in antiretroviral therapy. Nevirapine (NVR, Viramune®), efavirenz (EFV, Sustiva®), and delavirdine (DLV, Rescriptor®) are used in various combinations with nucleoside analogues and HIV protease inhibitors (1,2). In addition to the antiretroviral regimens, HIV patients are treated with a variety of other drugs. The resulting combinations may include antidepressants, antibiotics, analgesics, sedatives, etc., (3) and can render HIV patients prone to drug interactions. All NNRTIs are extensively metabolized by the cytochrome P450 system and have been reported to inhibit (DLV) or induce (NVR, EFV) hepatic enzymes (4–8) resulting in alterations in the pharmacokinetics of other drugs following coadministration of NNRTIs (9–11). In addition to the effects of a drug on hepatic metabolizing enzymes, interactions with drug transporters, especially P-glycoprotein (P-gp), the gene product of the human MDR1 gene, are receiving increasing attention. The 170 kD membrane protein P-gp is an ATP-dependent drug efflux pump that is constitutively expressed in several human tissues (epithelia of the small intestine, blood brain barrier endothelia, liver, kidney, testes, lymphocytes) (12,13) as well as in cancer cells (14). Factors contributing to potential P-gp interactions of a drug are its properties as a P-gp substrate, its potential to inhibit P-gp activity, and its ability to cause induction of P-gp expression after chronic exposure. A broad range of P-gp substrates and modulators has been identified. The HIV protease inhibitors are among them and are substrates as well as modulators of P-gp (15–17). Drug transporters are commonly studied in tissue culture and Caco-2 is a human colon adenocarcinoma cell line that resembles small intestinal epithelial cells in morphology and in the expression of various marker enzymes (18,19). This cell line is frequently used for studies modeling intestinal drug transport (20,21). For studies of P-gp induction, the human colon adenocarcinoma cell line LS180 has been useful after selection for elevated P-gp content (15,22,23). Using cell culture techniques, this study was designed to investigate the effects of the non-nucleoside HIV reverse transcriptase inhibitors nevirapine, efavirenz, and delavirdine on P-glycoprotein activity and expression to anticipate P-gp related drug-drug interactions associated with combination drug therapy.

MATERIALS AND METHODS

Chemicals

Drugs and chemicals were purchased from commercial sources, or were kindly provided by their pharmaceutical manufacturers. NVR, EFV, DLV, and ritonavir were extracted from their commercially available tablet formulations with methanol. It was assumed that the drugs were 100% soluble in methanol and that other components of the formulation were either not soluble in methanol, or were not pharmacologically active.

Cell Lines

The human colon adenocarcinoma cell line Caco-2 was kindly provided by Douglas Jefferson, Ph.D. (Tufts University School of Medicine and Tufts-New England Medical Center, Boston, Massachusetts) and used at passages 30–40. Caco-2 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium, GibcoBRL, Rockville, Maryland) supplemented with 10% fetal bovine serum, 0.1 mM non essential amino acids (GibcoBRL, Rockville, Maryland), 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

The human colon adenocarcinoma cell line LS180 (24) was obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were selected for elevated P-gp levels with increasing concentrations of vinblastine (1-

[1] Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, Massachusetts 02111.
[2] To whom correspondence should be addressed. (email: dj.greenblatt@tufts.edu)

0724-8741/02/0700-1038/0 © 2002 Plenum Publishing Corporation

2-4-8 ng/ml) and maintained at 4 ng/ml until initiation of induction experiments (23). The vinblastine selected cell line is denoted LS180V. LS180V cells were grown in MEM (Minimal Essential Media, GibcoBRL, Rockville, Maryland) supplemented with 10% fetal bovine serum, 0.1 mM non essential amino acids (GibcoBRL, Rockville, Maryland), 1 mM pyruvic acid, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 4 ng/ml vinblastine.

Transport Experiments

Caco-2 cells were seeded at $2 \times 10^4/cm^2$ in polycarbonate membrane transwell plates (2.5 cm diameter, 3 μm pore size) (Corning Costar Corp., Cambridge, Massachusetts) with 2.5 ml media in the basolateral (B) and 1.5 ml in the apical (A) chamber. Cells were grown in a humidified chamber (37°C, 5% CO_2) with media changes every 3–5 days. Transport experiments were conducted in confluent cells on days 16–20 post seeding. Transepithelial electrical resistance was >16000 ohms/cm^2, confirming high monolayer confluence. The P-gp index substrate Rh123, and the P-gp inhibitor verapamil as positive control, have been used previously in transport studies with Caco-2 cells (17,21,25). Methanolic solutions of drugs (except Rh123) were evaporated to dryness and reconstituted in Hank's Balanced Salt Solution (HBSS) (GibcoBRL, Rockville, Maryland) containing 10 mM HEPES, 4.2 mM $NaHCO_3$, and 0.5% DMSO. Solubility of the drugs in HBSS was verified by HPLC analysis. Peak heights were linear with drug concentration up to 250 μM NVR ($r^2 = 0.998$), 150 μM EFV ($r^2 = 0.984$), and 100 μM DLV ($r^2 = 0.976$). Rh123 was added as a concentrated methanolic solution, with final methanol concentration <0.5%.

P-gp Inhibition

HBSS 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% CO_2 for 180 min and 50 μl samples were taken from the chamber initially not containing Rh123. Samples were diluted with 500 μl methanol and fluorescence was determined at 500/550 nm (excitation/emission).

P-gp Substrates

Drug solutions (25 μM) were added to the A or B chamber, in presence and absence of verapamil (100 μM) in both chambers. Cells were incubated at 37°C, 5% CO_2 and 150 μl samples were taken after 90 min and 180 min from the chamber initially not containing the study drug. Samples were subjected to HPLC analysis as described below. Sampling times were chosen to assure detectable drug concentrations in both chambers even for non P-gp substrates. Caco-2 cells were viable and the monolayer stayed functional during the time course of the experiment as demonstrated by the linearity of Rh123 B to A transport between 60 min and 240 min ($r^2 = 0.988$).

P-gp Induction

LS180V cells were seeded at $2 \times 10^4/cm^2$ in tissue culture dishes (Corning Costar Corp., Cambridge, Massachusetts). Cells were grown in a humidified chamber (37°C, 5% CO_2) to 50% confluence (3 to 4 days), when media was replaced by vinblastine-free media containing the study drug and 0.5% DMSO. The known P-gp inducer ritonavir (10 μM) served as a positive control (15), and vehicle (media with 0.5% DMSO) served as a negative control. Experiments were done in triplicate. After 72 h, cells were either prepared for western blot analysis or subjected to Rh123 cell exclusion experiments as described below.

Sample Preparation and Western Blotting

After 72 h, the cells were lysed (0.5% deoxycholate, 1% triton-X, 1 mM PMSF in 0.05 M KH_2PO_4), sonicated and centrifuged. Supernatants were analyzed for protein concentration using the BCA protein assay (Pierce, Rockford, Illinois) and stored at –80°C until western blot analysis. Samples were separated by SDS-PAGE on 4-15% acrylamide gradient gels (Ready-Gel, BioRad, Hercule, California) and transferred to PVDF membranes (Immobilon P, Millipore, Bedford, Massachusetts). The 170 kD P-gp protein was detected using the monoclonal mouse anti-human P-gp (C219) primary antibody (Signet, Dedham, Massachusetts), a HRP linked sheep anti-mouse IgG secondary antibody (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey), and SuperSignal chemiluminiscence reagent (Pierce, Rockford, Illinois). Sample preparation, electrophoresis and transfer were performed at 4°C. Blots were quantified using integrated band density based on a calibration curve run together with each set of samples. Given the nonlinear relationship between the amount of protein loaded and integrated band density, the calibration curve allowed for comparison of *relative* P-gp contents between samples. Determination of *absolute* P-gp content requires a pure P-gp standard that is currently not available.

Rh123 Cell Exclusion

After 72 h, cells were washed five times with MEM (containing 10% calf serum and 0.5% DMSO) and supplied with vinblastine free media (1.5 ml/ well) with or without inhibitor (100 μM verapamil, 10–100 μM DLV). 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 five times with MEM (containing 10% calf serum and 0.5% DMSO). Cells were lysed for 30 min (10 min at 37°C, 20 min shaking at room temperature) and Rh123 concentrations in the lysate were quantified as described above. Determination of protein concentration demonstrated comparable cell density in each well. In similar experiments, LS180V cell morphology was evaluated microscopically as described by Perloff *et al.* (26) to assure cell viability. Furthermore, the Rh123 accumulation experiment verifies cell viability and cell membrane integrity itself. If LS180V cell membranes are compromised due to toxicity, the washing procedure during Rh123 accumulation experiments greatly reduces intracellular Rh123 (about 10-fold) and 100 μM VER has no reversal effect.

HPLC

NNRTIs and verapamil were separated using a 3.9 × 300 mm μBondapak (C18) 10 μm column, (Waters Associates, Milford, Massachusetts) at 40°C and UV absorbance was

monitored at 232 nm (NVR) or 245 nm (EFV, DLV). The mobile phase consisted of 21% acetonitrile and 79% 0.05 M KH_2PO_4 (pH 3.5) for NVR, 45% acetonitrile and 55% 0.05 M KH_2PO_4 (pH 4.5) for EFV, and 45% acetonitrile and 55% 0.05 M KH_2PO_4 (pH 5.5) for DLV and was delivered at a flow rate of 2.4 ml/min.

Data Analysis

IC_{50} values were determined by nonlinear least squares regression (SigmaPlot 4.01, SPSS Inc.). Integrated density of western blot bands was determined by computer image analysis (Scion Image 4.02, Scion Corp., Frederick, Maryland). For western blot and cell exclusion studies, values for active drug exposure groups were expressed as a ratio vs. the mean vehicle control value. Mean ratios for each group were assessed for differences from unity using Student's *t*-test.

RESULTS

NNRTIs as Transport Substrates

In a transwell system, transport of NNRTIs across a Caco-2 cell monolayer was evaluated in both directions, basolateral to apical (B to A) and apical to basolateral (A to B). The three NNRTIs showed no differential transport, compared to a 17-fold difference observed for the positive control Rh123, a known P-gp substrate (Table I). The P-gp inhibitor verapamil (100 μM) reduced Rh123 B to A transport to 10% of control, but did not affect transport of NVR, EFV, or DLV across Caco-2 cells (Table I). Together these findings suggest that none of the NNRTIs is subject to transport that can be distinguished from passive diffusion in this Caco-2 cell system.

Table I. Transport of NNRTIs and Rh123 (positive control) across Caco-2 Cell Monolayers in a Transwell System in Presence and Absence of Verapamil (100 μM)

	B → A[a] (nmol/cm^2)	A → B[b] (nmol/cm^2)
Nevirapine (25 μM)		
– verapamil	3.2 (0.24)	3.3 (0.08)
+ verapamil	3.3 (0.38)	3.9 (0.33)
Efavirenz (25 μM)		
– verapamil	1.9 (0.26)	1.5 (0.27)
+ verapamil	1.6 (0.18)	1.9 (0.08)
Delavirdine (25 μM)		
– verapamil	4.1 (0.63)	3.4 (0.31)
+ verapamil	4.8 (0.59)	4.4 (0.57)
Rhodamine123 (5 μM)		
– verapamil	0.33 (0.013)	0.02 (0.008)[c]
+ verapamil	0.04 (0.005)[d]	0.01 (0.001)[c]

Note: Values in nmol of drug transported per square cm of membrane area after a 180 min incubation at 37°C. Data represent means of triplicate samples, standard deviations in parenthesis.

[a] Basolateral to apical chamber.

[b] Apical to basolateral chamber.

[c] Statistically significant compared to the corresponding B → A transport ($P < 0.05$, ANOVA).

[d] Statistically significant compared to the corresponding sample without verapamil ($P < 0.05$, ANOVA).

Inhibition of Rh123 Transport by NNRTIs:

NVR, EFV, and DLV were evaluated for their potential to inhibit P-gp mediated basolateral to apical Rh123 transport across Caco-2 monolayers associated with acute exposure to the drugs (90 and 180 min incubation time). Toxicity of 100 μM EFV in Caco-2 cells was evident as cells were detached from the membrane and drug diffusion in both directions was unrestricted. No such effect was observed with 3–30 μM EFV.

NVR (100 μM), and EFV (30 μM) did not affect Rh123 transport. DLV (100 μM) reduced Rh123 transport to 15% of control, an effect comparable to that of the known P-gp inhibitor verapamil (100 μM) that caused a reduction to 10% of control (Fig. 1). Rh123 transport inhibition by DLV was concentration-dependent (Fig. 1, insert), with an IC_{50} value of 30 μm.

Induction of P-gp Expression by NNRTIs

NVR, EFV, and DLV were evaluated for their ability to induce P-gp expression in LS180V cells after extended (72 h) exposure. Western blot analysis of membrane preparations of the exposed cells revealed a concentration-dependent increase in immunoreactive P-gp after NVR exposure. At 100 μM, NVR caused a 3.5-fold increase in P-gp expression over vehicle control (Fig. 2). This effect was comparable to the 4.3-fold induction caused by 10 μM ritonavir, a known P-gp inducer used as a positive control (15). The induction caused by EFV (1.75-fold) and DLV (2.35-fold) was less substantial and also concentration-dependent. Toxicity to the cells and limited solubility in tissue culture media precluded the use of higher drug concentrations.

Induction of P-gp Activity by NNRTIs

Extended exposure (72 h) to NVR (100 μM) and EFV (30 μM) reduced Rh123 accumulation in LS180V cells to 72% and 81% of vehicle control, respectively. DLV (100 μM) reduced Rh123 cell accumulation to 85% of control. The positive control ritonavir (10 μM) caused a decrease in Rh123 uptake to 49% of control. Addition of the P-gp inhibitor verapamil reversed the effect in both controls and drug treated cells, increasing accumulation of Rh123 about 3-fold (Fig. 3). The data corresponds well to the western blot results for NVR, EFV and DLV, all of which caused a smaller inductive effect than ritonavir. Together, these results indicate that after extended exposure to the NNRTIs or ritonavir, active Rh123 efflux from the cell is increased as a result of P-gp induction.

Combined Induction and Inhibition of P-gp Activity in LS180V Cells

Cells preexposed to NVR (100 μM) or ritonavir (10 μM) had decreased intracellular Rh123 compared to DMSO control, consistent with transporter induction (Fig. 4, values for DLV = 0). Acute exposure to increasing concentrations of DLV in DMSO control cells produced an increase in intracellular Rh123, consistent with inhibition of transport function. However DLV produced at most a small degree of reversal of Rh123 intracellular accumulation in cells induced by prior exposure to NVR or ritonavir (Fig. 4).

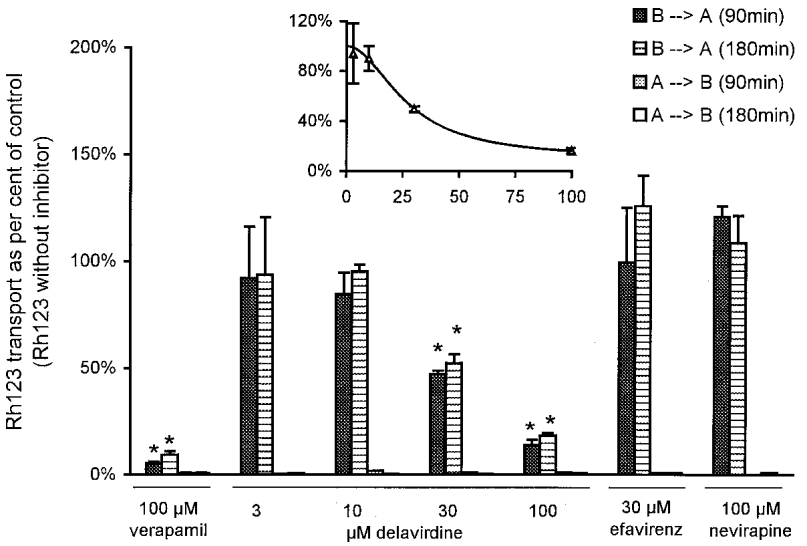

Fig. 1. Inhibition of P-gp mediated rhodamine123 transport across Caco-2 monolayers by delavirdine, efavirenz, and nevirapine. Data represent Rh123 transport in the presence of inhibitors as a percent of the uninhibited control. Values are means of triplicate samples with standard deviation indicated by error bars. The known P-gp inhibitor verapamil (100 μM) served as a positive control. Due to toxicity to Caco-2 cells, efavirenz could not be tested at 100 μM. (A) Apical chamber and (B) basolateral chamber. Asterisk (*) indicates a statistically significant ($P < 0.05$) difference from 100% using the Student's two-tailed *t*-test. (Insert) Concentration dependent P-gp inhibition by delavirdine in Caco-2 cells. Data represent Rh123 transport as a percent of the uninhibited control, mean of the 90 and 180 min incubations. The line represents the function obtained by nonlinear regression (IC_{50} = 30 μM).

DISCUSSION

The present *in vitro* study showed that NNRTIs can significantly modulate P-glycoprotein activity and expression, based on transport of the index substrate Rh123. The NNRTIs themselves did not appear to be substrates for transport, as transport can be distinguished from passive diffusion in the cell model. DLV was found to be an inhibitor of Rh123 transport at concentrations within the range of total plasma DLV concentrations seen *in vivo* (27). All three NNRTIs were able to cause induction in P-gp expression and activity after extended exposure at concentrations in the range of 30–100 μM, with the inductive effect of NVR being quantitatively the largest. Although plasma concentrations of NNRTIs are usually <25 μM (10), the concentrations achieved in the intestine after oral administration of usual therapeutic doses of NNRTIs will transiently exceed plasma concentrations and may reach millimolar levels.

Considering that antiretroviral drugs are used almost always in combination therapy, the effects of P-gp inhibitors on LS180V cells pretreated with P-gp inducing drugs was studied. DLV increased Rh123 accumulation in uninduced control LS180V cells in a concentration dependent manner (Fig. 4), an effect similar to the inhibition of Rh123 transport observed in Caco-2 cells (Fig. 1). In LS180V cells exposed for 72 h to NVR or ritonavir (100 μM or 10 μM, respectively), acute exposure to DLV at 30 μM only partially reversed the decrease in intracellular accumulation of Rh123 related to P-gp induction by NVR. DLV at 100 μM had no detectable effect, and DLV had no evident reversal effect on cells induced by exposure to ritonavir. The findings suggest that the effects of P-gp induction by NVR or ritonavir largely outweigh the inhibitory activity of acute exposure to DLV in this model. The fact that the more potent P-gp inhibitor verapamil did reverse the observed induction (Fig. 3) demonstrates that the relative potencies of the study compounds influence the net outcome of concomitant induction and inhibition of P-gp.

The effects of NNRTIs on Rh123 transport did parallel their previously reported effects on CYP3A, with DLV being a potent inhibitor of both CYP3A (6,7) and the transporter. All three compounds induce CYP3A (8–11) and P-gp, with NVR being the most potent. Such coincident effects on P-gp and CYP3A have been reported for a variety of compounds (22,28–30), and it appears that dissociation of inhibitory potencies for P-gp and CYP3A4 remains an ongoing challenge in drug development (31,32) although co-regulation of CYP3A and transporter activity is not invariable (33,34).

We used different cell lines for substrate/inhibition and induction studies because of different morphologic characteristics of LS180 and Caco-2 cells. Evaluation of directional transport and its inhibition require a cell line that forms a tight monolayer to facilitate transwell experiments. Caco-2 cell monolayers become confluent and minimize passive paracellular diffusion between the chambers, making Caco-2 cells one of the most frequently used cell line for this type of study (18–21). LS180 cell agglomerates, however, are more loose and often detach from the culture dish after reaching about 70% confluency, which is not sufficient for transwell experiments. Induction studies, on the other hand, have been less successful with Caco-2 cells because of the late onset of P-gp expression in this cell line. Caco-2 cells do not express substantial amounts of P-gp until confluency (18), and the ideal time point for studies of induction is not clear. In contrast,

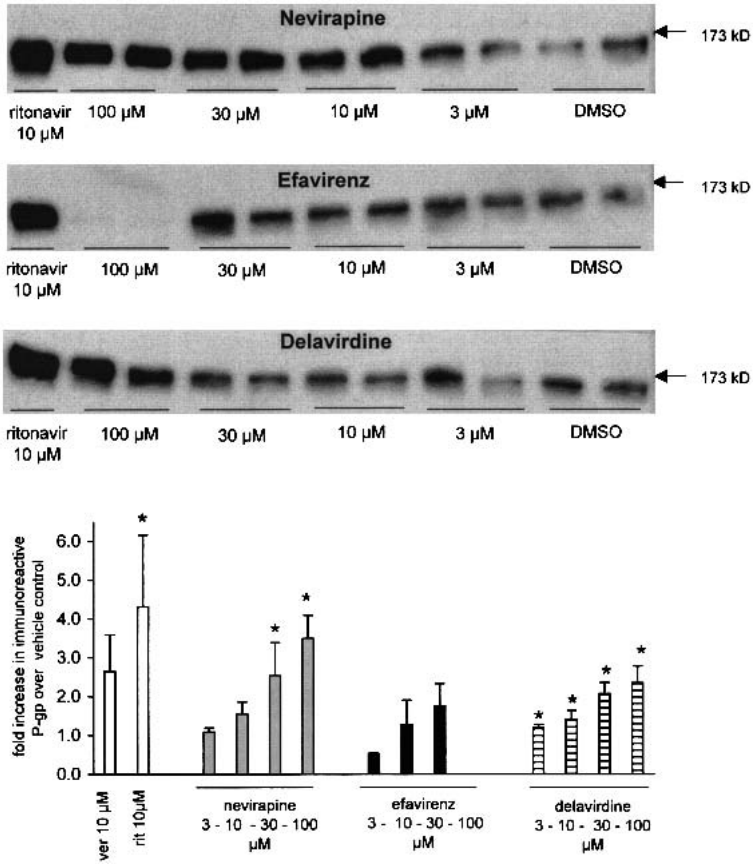

Fig. 2. P-gp expression in LS180V cells after 3 days exposure to nevirapine, efavirenz, and delavirdine was assessed by western blot analysis. Blots were quantified using integrated band density based on a calibration curve run together with each set of samples. Shown are representative western blots produced after quantitative analysis for illustration purposes only. Data represent immunoreactive P-gp content relative to vehicle control (LS180 media with 0.5% DMSO) (n = 3). The known P-gp inducer ritonavir (10 µM) served as a positive control. Efavirenz 100 µM was toxic to LS180V cells. Asterisk (*) indicates a significant ($P < 0.05$) difference from the mean vehicle control (1.0-fold increase) using the Student's one-tailed *t*-test.

previous studies have demonstrated that P-gp expression in LS180 cells is inducible by a number of compounds (15,22,23). Although Caco-2 cells have substantially lower P-gp levels than cell lines such as NIH3T3/MDR1 or LS180V (35), there is sufficient evidence that Caco-2 cells closely resemble human (and rat) small intestinal mucosa in P-gp expression as well as in transport kinetics of different model substrates (36,37) and are therefore considered to be a reasonable model for intestinal transport (20,37,38). It seems possible however, that P-gp mediated transport of lipophilic substances, which may be quantitatively important in other models or *in vivo*, may not be evident in the present model due to the greater quantitative importance of passive diffusion. Whereas two different models were used in this study, the respective results are not combined to make quantitative predictions for *in vivo* drug interactions, but rather taken as separate indicators of potential P-gp inhibition and induction *in vivo*, providing valuable information for the design of future clinical studies.

Evaluation of transporters other than P-gp, including MRP, LRP, and dipeptide transporters (39) in Caco-2 cells, require the use of index compounds that are relatively specific for the transporter studied. The fluorescent dye Rh123 is frequently used as a P-gp index substrate. A drug screen of the National Cancer Institute in 58 different cell lines showed a good correlation of Rh123 transport with P-gp expression, while no correlation was found with the expression of MRP in these cell lines (25). Additional experiments with our Caco-2 cell line (data not shown) demonstrated that 2 mM probenecid and 100 µM indomentacin, both known inhibitors of MRP1, did not inhibit Rh123 transport thereby suggesting that MRP1 does not contribute importantly to Rh123 transport. Verapamil is widely used as an inhibitor of P-gp, and its inhibitory potency has been demonstrated in MDR-1 transfected MDCK cells that constitutively express only low levels of transport proteins and metabolizing enzymes (40). However, verapamil may also inhibit transporters other than P-gp (41). We have previously observed that induction by ritonavir exhibits some variability in susceptibility to verapamil inhibition, whereas induction itself is less variable. The possibility of differential effects on transporters other than P-gp by the study drugs can not be excluded and may contribute to the

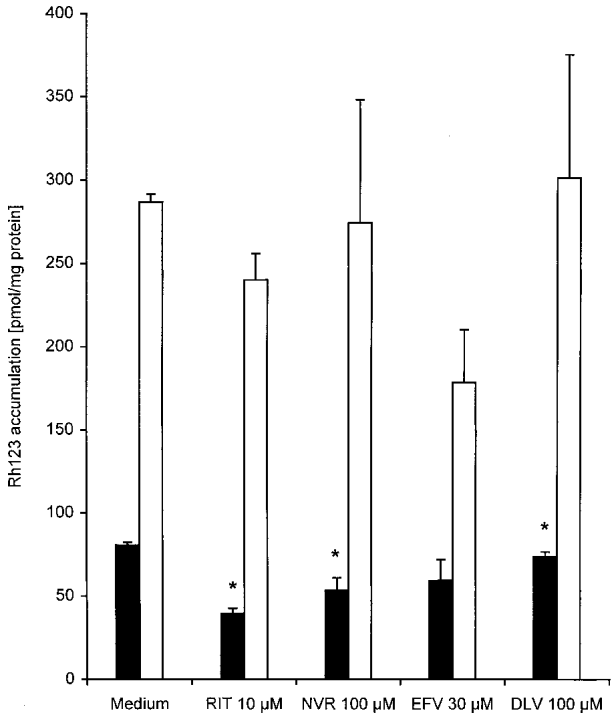

Fig. 3. Intracellular Rh123 accumulation in LS180V cells after 3 days exposure to nevirapine, efavirenz, and delavirdine and reversal of the effect by acute verapamil (100 μM). (shaded bars) Rh123 intracellular accumulation in the absence of verapamil; (open bars) Rh123 intracellular accumulation with acute exposure to verapamil. Values are means of triplicate samples with standard deviation indicated by error bars. The known P-gp inducer ritonavir (10 μM) served as a positive control. Asterisk (*) indicates a significant ($P < 0.05$) difference from the mean control value using Student's one-tailed *t*-test.

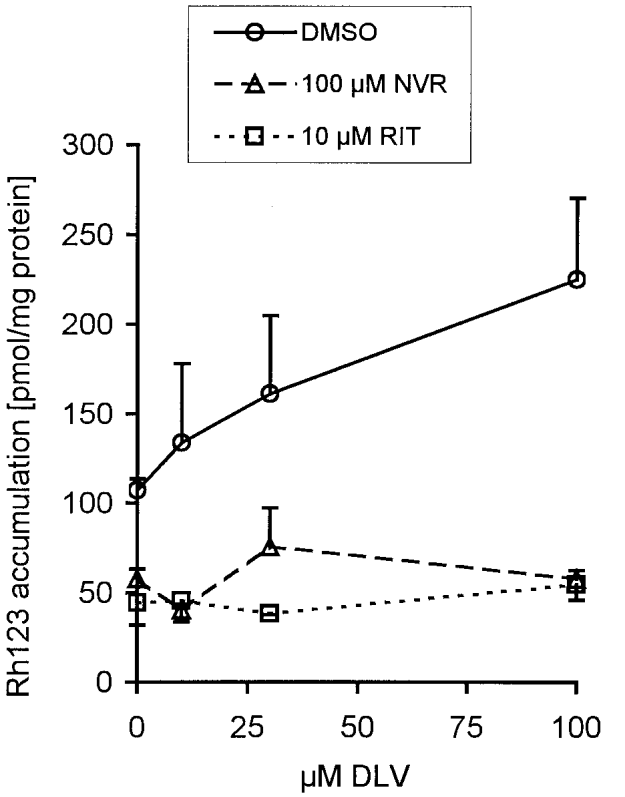

Fig. 4. Combined effects of P-gp induction and inhibition by NNRTIs on intracellular Rh123 accumulation in LS180V cells. Data represent Rh123 accumulation in cells acutely exposed to varying concentrations of DLV. Cells were previously exposed for 72 h to DMSO (control) or to P-gp inducing drugs. NVR, ritonavir (RIT)]. Values are means of triplicate samples with standard deviation indicated by error bars.

variable effects of VER on Rh123 accumulation in induced cells.

Our data suggest a potential for NNRTIs to cause drug interactions at the level of drug transport in addition to their effects on CYP enzyme activity. Several studies investigating the pharmacokinetics of NNRTIs in combination with HIV protease inhibitors described significant drug interactions. Increased plasma concentrations of protease inhibitors following DLV coadministration, as well as decreased plasma levels seen with NVR (10), have been attributed to CYP3A4 inhibition and induction, respectively. Although all protease inhibitors are extensively metabolized by CYP3A4 (42,43), the magnitude of the reported interactions varies considerably between drugs, with increases in AUC following DLV coadministration ranging from 70% for ritonavir up to 400% for saquinavir (10). Since protease inhibitors are not only metabolized by CYP3A4 but are also substrates of P-gp (44), these variable effects may be the result of concomitant modulation of both P-gp and CYP3A4 by NNRTIs. In this case, different affinities of the protease inhibitors as P-gp substrates could account for the variation in effects observed.

In conclusion, NVR, EFV, and DLV are not evidently substrates for transport in Caco-2 cell monolayers. DLV is an inhibitor of Rh123 transport *in vitro* at clinically relevant drug concentrations, while NVR, and to a lesser extent EFV and DLV, increase activity and expression of P-gp in cell culture. Drug interaction studies testing NNRTIs together with drugs that are substrates of P-gp but not CYP3A (e.g., digoxin, fexofenadine) are required to evaluate the clinical relevance of these *in vitro* findings.

ACKNOWLEDGMENTS

E.S. was the recipient of a HSP III doctoral grant from the German Academic Exchange Service (DAAD).This work was supported by grants DA-13209, MH-58435, DK/AI-58496, DA-13834, MH-01237, MH-34223, and DA-05258 from the U.S. Department of Health and Human Services.

REFERENCES

1. E. De Clercq. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. *Antiviral Res.* **38**:153–179 (1998).
2. D. V. Havlir and J. M. Lange: New antiretrovirals and new combinations. *AIDS* **12SupplA**:S165–S174 (1998).
3. I. Fogelman, L. Lim, R. Bassett, P. Volberding, M. A. Fischl, K. Stanley, and D. J. Cotton. Prevalence and patterns of use of concomitant medications among participants in three multicenter human immunodeficiency virus type I clinical trials. AIDS Clinical Trials Group (ACTG). *J. Acquir. Immune Defic. Syndr.* **7**: 1057–1063 (1994).
4. P. Riska, M. Lamson, T. MacGregor, J. Sabo, S. Hattox, J. Pav, and J. Keirns. Disposition and biotransformation of the antiretroviral drug nevirapine in humans. *Drug Metab. Dispos.* **27**:895–901 (1999).

5. C. L. Cheng, D. E. Smith, P. L. Carver, S. R. Cox, P. B. Watkins, D. S. Blake, C. A. Kauffman, K. M. Meyer, G. L. Amidon, and P. L. Stetson. Steady-state pharmacokinetics of delavirdine in HIV-positive patients: effect on erythromycin breath test. *Clin. Pharmacol. Ther.* **61**:531–543 (1997).
6. R. L. Voorman, S. M. Maio, N. A. Payne, Z. Zhao, K. A. Koeplinger, and X. Wang. Microsomal metabolism of delavirdine: evidence for mechanism-based inactivation of human cytochrome P450 3A. *J. Pharmacol. Exp. Ther.* **287**:381–388 (1998).
7. L. L. von Moltke, D. J. Greenblatt, B. W. Granda, G. M. Giancarlo, S. X. Duan, J. P. Daily, J. S. Harmatz, and R. I. Shader. Inhibition of human cytochrome P450 isoforms by non-nucleoside reverse transcriptase inhibitors. *J. Clin. Pharmacol.* **41**:85–91 (2001).
8. J. C. Adkins and S. Noble. Efavirenz. *Drugs* **56**:1055–1064 (1998).
9. F. L. Altice, G. H. Friedland, and E. L. Cooney. Nevirapine induced opiate withdrawal among injection drug users with HIV infection receiving methadone. *AIDS* **13**:957–962 (1999).
10. M. Barry, F. Mulcahy, C. Merry, S. Gibbons, and D. Back. Pharmacokinetics and potential interactions amongst antiretroviral agents used to treat patients with HIV infection. *Clin. Pharmacokinet.* **36**:289–304 (1999).
11. X. Duval, V. Le Moing, C. Longuet, C. Leport, J. L. Vilde, C. Lamotte, G. Peytavin, and R. Farinotti. Efavirenz-induced decrease in plasma amprenavir levels in human immunodeficiency virus-infected patients and correction by ritonavir. *Antimicrob. Agents Chemother.* **44**:2593 (2000).
12. I. Sugawara, I. Kataoka, Y. Morishita, H. Hamada, T. Tsuruo, S. Itoyama, and S. Mori. Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Res.* **48**:1926–1929 (1988).
13. C. Cordon-Cardo, J. P. O'Brien, D. Casals, L. Rittman-Grauer, J. L. Biedler, M. R. Melamed, and J. R. Bertino. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* **86**:695–698 (1989).
14. V. Ling, N. Kartner, T. Sudo, L. Siminovitch, and J. R. Riordan. Multidrug-resistance phenotype in Chinese hamster ovary cells. *Cancer Treat. Rep.* **67**:869–874 (1983).
15. M. D. Perloff, L. L. von Moltke, J. M. Fahey, J. P. Daily, and D. J. Greenblatt. Induction of P-glycoprotein expression by HIV protease inhibitors in cell culture. *AIDS* **14**:1287–1289 (2000).
16. R. B. Kim, M. F. Fromm, C. Wandel, B. Leake, A. J. Wood, D. M. Roden, and G. R. Wilkinson. The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J. Clin. Invest.* **101**:289–294 (1998).
17. L. Profit, V. A. Eagling, and D. J. Back. Modulation of P-glycoprotein function in human lymphocytes and Caco-2 cell monolayers by HIV-1 protease inhibitors. *AIDS* **13**:1623–1627 (1999).
18. M. Pinto, S. Robine-Leon, M. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. Enterocyte-like differentiation and polarisation of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47**:323–330 (1983).
19. A. Quaroni and J. Hochman. Developement of intestinal cell culture models for drug transport and metabolism studies. *Adv. Drug Deliv. Rev.* **22**:3–52 (1996).
20. P. Artursson, K. Palm, and K. Luthman. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **22**:67–84 (1996).
21. R. Yumoto, T. Murakami, Y. Nakamoto, R. Hasegawa, J. Nagai, and M. Takano. Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. *J. Pharmacol. Exp. Ther.* **289**:149–155 (1999).
22. E. G. Schuetz, W. T. Beck, and J. D. Schuetz. Modulators and substrates of P-glycoprotein and cytochrome P4503A coordinately up-regulate these proteins in human colon carcinoma cells. *Mol. Pharmacol.* **49**:311–318 (1996).
23. C. E. Herzog, M. Tsokos, S. E. Bates, and A. T. Fojo. Increased mdr-1/P-glycoprotein expression after treatment of human colon carcinoma cells with P-glycoprotein antagonists. *J. Biol. Chem.* **268**:2946–2952 (1993).
24. B. H. Tom, L. P. Rutzky, M. M. Jakstys, R. Oyasu, C. I. Kaye, and B. D. Kahan. Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro* **12**:180–191 (1976).
25. J. S. Lee, K. Paull, M. Alvarez, C. Hose, A. Monks, M. Grever, A. T. Fojo, and S. E. Bates. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. *Mol. Pharmacol.* **46**:627–638 (1994).
26. M. D. Perloff. L. L. Von Moltke, J. E. Marchand, and D. J. Greenblatt: Ritonavir induces P-glycoprotein expression, multidrug resistance-associated protein (MRP1) expression, and drug transporter-mediated activity in a human intestinal cell line. *J. Pharm. Sci.* **90**:1829–1837 (2001).
27. W. W. Freimuth. Delavirdine mesylate, a potent non-nucleoside HIV-1 reverse transcriptase inhibitor. *Adv. Exp. Med. Biol.* **394**: 279–289 (1996).
28. T. Tateishi, H. Nakura, M. Asoh, M. Watanabe, M. Tanaka, T. Kumai, and S. Kobayashi. Multiple cytochrome P-450 subfamilies are co-induced with P-glycoprotein by both phenothiazine and 2-acetylaminofluorene in rats. *Cancer Lett.* **138**:73–79 (1999).
29. E. L. Gujaeva, V. A. Kobliakov, T. N. Zabotina, E. J. Rybalkina, and A. A. Stravrovskaya. Coordinated regulation of P-glycoprotein activity and cytochrome P-4501A induction in sublines of rat hepatoma McA RH7777 cells with different levels of colchicine resistance. *Membr. Cell Biol.* **12**:481–488 (1998).
30. L. L. von Moltke, D. J. Greenblatt, J. M. Grassi, B. W. Granda, S. X. Duan, S. M. Fogelman, J. P. Daily, J. S. Harmatz, and R. I. Shader. Protease inhibitors as inhibitors of human cytochromes P450: high risk associated with ritonavir. *J. Clin. Pharmacol.* **38**: 106–111 (1998).
31. V. J. Wacher, C. Y. Wu, and L. Z. Benet. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol. Carcinog.* **13**:129–134 (1995).
32. C. Wandel, R. B. Kim, S. Kajiji, P. Guengerich, G. R. Wilkinson, and A. J. Wood. P-glycoprotein and cytochrome P-450 3A inhibition: dissociation of inhibitory potencies. *Cancer Res.* **59**:3944–3948 (1999).
33. D. Runge, C. Kohler, V. E. Kostrubsky, D. Jager, T. Lehmann, D. M. Runge, U. May, D. B. Stolz, S. C. Strom, W. E. Fleig, and G. K. Michalopoulos. Induction of cytochrome P450 (CYP)1A1, CYP1A2, and CYP3A4 but not of CYP2C9, CYP2C19, multidrug resistance (MDR-1) and multidrug resistance associated protein (MRP-1) by prototypical inducers in human hepatocytes. *Biochem. Biophys. Res. Commun.* **273**:333–341 (2000).
34. R. B. Kim, C. Wandel, B. Leake, M. Cvetkovic, M. F. Fromm, P. J. Dempsey, M. M. Roden, F. Belas, A. K. Chaudhary, D. M. Roden, A. J. Wood, and G. R. Wilkinson. Interrelationship between substrates and inhibitors of human CYP3A and P-glycoprotein. *Pharm. Res.* **16**:408–414 (1999).
35. S. Ibrahim, J. Peggins, A. Knapton, T. Licht, and A. Aszalos. Influence of antipsychotic, antiemetic, and Ca(2+) channel blocker drugs on the cellular accumulation of the anticancer drug daunorubicin: P-glycoprotein modulation. *J. Pharmacol. Exp. Ther.* **295**:1276–1283 (2000).
36. R. H. Stephens, C. A. O'Neill, A. Warhurst, G. L. Carlson, M. Rowland, and G. Warhurst. Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human intestinal epithelia. *J. Pharmacol. Exp. Ther* **296**:584–591 (2001).
37. V. D. Makhey, A. Guo, D. A. Norris, P. Hu, J. Yan, and P. J. Sinko. Characterization of the regional intestinal kinetics of drug efflux in rat and human intestine and in Caco-2 cells. *Pharm. Res.* **15**:1160–1167 (1998).
38. P. Artursson and J. Karlsson. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun.* **175**:880–885 (1991).
39. C. A. Bailey, P. Bryla, and A. W. Malick. The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. *Adv. Drug Deliv. Rev.* **22**:85–103 (1996).
40. A. Braun, S. Hammerle, K. Suda, B. Rothen-Rutishauser, M. Gunthert, S. D. Kramer, and H. Wunderli-Allenspach. Cell cultures as tools in biopharmacy. *Eur. J. Pharm. Sci.* **11Suppl2**:S51–S60 (2000).
41. A. Aszalos, K. Thompson. J. J. Yin, and D. D. Ross: Combinations of P-glycoprotein blockers, verapamil, PSC833, and cremophor act differently on the multidrug resistance associated pro-

tein (MRP) and on P-glycoprotein (Pgp). *Anticancer Res.* **19**: 1053–1064 (1999).
42. T. Koudriakova, E. Iatsimirskaia, I. Utkin, E. Gangl, P. Vouros, E. Storozhuk, D. Orza, J. Marinina, and N. Gerber. Metabolism of the human immunodeficiency virus protease inhibitors indinavir and ritonavir by human intestinal microsomes and expressed cytochrome P4503A4/3A5: mechanism-based inactivation of cytochrome P4503A by ritonavir. *Drug Metab. Dispos.* **26**:552–561 (1998).
43. M. E. Fitzsimmons and J. M. Collins. Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small-intestinal cytochrome P4503A4: potential contribution to high first-pass metabolism. *Drug Metab. Dispos.* **25**:256–266 (1997).
44. C. G. Lee, M. M. Gottesman, C. O. Cardarelli, M. Ramachandra, K. T. Jeang, S. V. Ambudkar, I. Pastan, and S. Dey. HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* **37**:3594–3601 (1998).