Potential of novel antiretrovirals to modulate expression and function of drug transporters in vitro

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Objectives: The chemokine receptor antagonists maraviroc and vicriviroc and the integrase inhibitors elvitegravir and raltegravir are novel antiretroviral agents for the treatment of HIV-1 infections. ATP-binding cassette (ABC) transporters as modulators of the effectiveness and safety of therapy can mediate viral resistance and drug–drug interactions. To expand knowledge on drug–drug interactions of these antiretrovirals we investigated whether these compounds are substrates, inhibitors or inducers of important ABC transporters.

Methods: We evaluated P-glycoprotein (P-gp/ABCB1) inhibition by the calcein assay in P388/dx and L-MDR1 cells, breast cancer resistance protein (BCRP/ABCG2) inhibition in MDCKII-BCRP cells by pheophorbide A efflux, and inhibition of the multidrug resistance-associated protein 2 (MRP2/ABCC2) by using the MRP2 PRE-DIVEZ™ Vesicular Transport Kit. Substrate characteristics were evaluated by growth inhibition assays in MDCKII cells overexpressing particular ABC transporters. Induction of transporters was quantified by real-time RT–PCR in LS180 cells and for ABCB1 also at the functional level.

Results: Elvitegravir and vicriviroc inhibited ABCB1 in P388/dx and L-MDR1 cells (f2 values 1.9 ± 0.2 μmol/L and 8.5 ± 3.6 μmol/L, respectively). The IC50 for ABCG2 inhibition was 15.7 ± 5.7 μmol/L for elvitegravir and 236.7 ± 93.3 μmol/L for vicriviroc. Raltegravir and maraviroc showed no evidence of ABCB1 or ABCG2 inhibition. Maraviroc and vicriviroc stimulated ABCC2 transport function. Growth inhibition assays suggest that elvitegravir, raltegravir and vicriviroc are substrates of ABCB1. Induction assays demonstrate that mRNA expression of several ABC transporters is induced by these antiretrovirals in LS180 cells.

Conclusions: The new antiretrovirals bear the potential to modulate expression and function of several ABC transporters, with elvitegravir revealing the highest interaction potential.

Keywords: elvitegravir, maraviroc, raltegravir, vicriviroc, drug interactions

Introduction

Highly active antiretroviral therapy (HAART), consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) or boosted protease inhibitor (PI) individualized for the patient, is the recommended first-line treatment of HIV-1 infections1 and provides sustained control of virus replication in many patients.2 Treatment failure, however, can occur for clinical, immunological and virological reasons,3 multidrug resistance (MDR) being one of the major obstacles. New treatment options for salvage therapy are therefore of considerable importance, and several new antiretrovirals have recently been approved or are currently in clinical development. Raltegravir represents the first drug of the new class of integrase inhibitors, which target viral integrase and inhibit integration of the viral DNA copy into the DNA of host cells.3 Raltegravir is approved for the treatment of HIV-1 infections in combination with other antiretroviral medicinal products in adult patients and represents, in addition to NRTIs, NNRTIs and PIs, a new option in therapy-naive patients. Elvitegravir is also an integrase inhibitor and is currently being tested in Phase III clinical studies. Maraviroc, the first approved drug of the class of CC chemokine receptor 5 (CCR5) antagonists, binds to the human chemokine receptor CCR5 and prevents binding of viral gp120 to co-receptor CCR5 and hence entry of the viral capsid into the host cell.3 Maraviroc has received approval for add-on treatment of therapy-experienced adult patients infected with only CCR5-tropic HIV-1. The CCR5 antagonist vicriviroc is currently...
being tested in treatment-naive patients in an ongoing Phase II clinical study.

Pharmacokinetic drug–drug interactions are a critical factor for treatment response to antiretrovirals. Interactions may lower drug concentrations to subtherapeutic concentrations, thus cause treatment failure and promote viral resistance. In contrast, interactions increasing drug exposure may augment toxicity. Main mechanisms of interaction in antiretroviral therapy involve efflux transporters, uptake transporters and the drug-metabolizing cytochrome P450 enzymes (CYPs). Crucial efflux transporters are the ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2), which have been identified as major sites of antiretroviral drug interactions. Relevant uptake transporters comprise the organic anion-transporting polypeptides/solute carrier proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2), which have been identified as major sites of antiretroviral drug interactions. Relevant uptake transporters comprise the organic anion-transporting polypeptides/solute carrier proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2), which have been identified as major sites of antiretroviral drug interactions. Relevant uptake transporters comprise the organic anion-transporting polypeptides/solute carrier proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2), which have been identified as major sites of antiretroviral drug interactions.

Data on interactions of elvitegravir, maraviroc, raltegravir and vicriviroc are sparse. Elvitegravir is metabolized by CYP3A enzymes and glucuronidated by UGT1A1 and UGT1A3. Maraviroc is suggested to be a substrate of ABCB1 and CYP3A4. If maraviroc is given in combination with CYP3A4 inducers such as efavirenz, the maraviroc dose needs to be increased. In combination with an inducer and an inhibitor of CYP3A4, such as protease inhibitors, the net effect is inhibition and dose reduction might be necessary. Raltegravir is not metabolized by CYP enzymes and to date there is no evidence for relevant induction or inhibition of drug-metabolizing enzymes. Vicriviroc is metabolized primarily by CYP3A4 and to a small extent by CYP3A5 and CYP2C9. These new antiretrovirals clearly offer a new seminal possibility for salvage therapy of HIV patients. However, thus far data on their drug–drug interaction potential are insufficient, although crucial for maximum safety and effectiveness. Indeed, little attention has been paid to the substrate characteristics of new antiretrovirals regarding ABCB1, ABCG2 and ABCCs and their potential to inhibit or induce drug transporters. We therefore assessed whether elvitegravir, raltegravir, maraviroc and vicriviroc are substrates of ABCB1, ABCG2, ABCC1, ABCC2 or ABCC3 by performing cellular resistance (proliferation) assays. Additionally, we tested their inhibitory potential for ABCB1, ABCG2 and ABCC2. Moreover, we investigated whether these antiretrovirals induce ABC transporters, OATPs/SLCOs, CYPs and the pregnane X receptor (PXR) by exposure of human adenocarcinoma LS180 cells, which are a surrogate for the major site of drug interactions and a well-established induction model.

Materials and methods

Materials

Culture media, medium supplements, DMSO, Hanks balanced salt solution (HBSS), rhodamine 123 and PBS were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum (FCS) was purchased from PAA (Pasching, Austria) and Triton X-100 from Roche Applied Science (Mannheim, Germany). Crystal Violet and rifampicin were purchased from AppliChem (Darmstadt, Germany). Methanol was obtained from Roth (Karlsruhe, Germany). Cell culturing bottles were supplied by Greiner (Frickenhausen, Germany), and 96-well microtitre plates were supplied by Nunc (Wiesbaden, Germany). Collagen R was obtained from Serva (Heidelberg, Germany). Phosphoribose A (Pha) was purchased from Frontier Scientific Europe (Carnforth, UK) and calcein acetonidemethylester (calcein-AM) from Invitrogen (Karlsruhe, Germany). MK571 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). RNeasy Mini-Kit was obtained from Qiagen (Hilden, Germany). Fumitremorgin C (FTC) was kindly provided by the National Cancer Institute (Rockville, USA). LY335979 (zosuquidar) was a kind gift of Eli Lilly (Bad Homburg, Germany). Elvitegravir and vicriviroc were purchased from Selleck Chemicals LLC (Houston, TX, USA). Maraviroc was kindly provided by Pfizer GmbH (Berlin, Germany) and raltegravir by Merck Sharp & Dohme GmbH (Haar, Germany).

Stock solutions of test compounds

Stock solutions of vicriviroc (10 mmol/L) and raltegravir (10 mmol/L) were prepared in sterile water. Stock solutions of maraviroc (10 mmol/L) and elvitegravir (10 mmol/L) were prepared in DMSO. The maximum concentration of DMSO was limited to concentrations that have previously been demonstrated to have no effect in the respective assay (1% in inhibition and growth inhibition assays and 0.1% in induction assays).

Cell lines

MDCKII, MDCKII-BCRP, MDCKII-MDR1, MDCKII-MRP1, MDCKII-MRP2 and MDCKII-MRP3 cells

MDCKII-BCRP cells were generated by stable transfection with human full-length wild-type cDNA and green fluorescent protein. MDCKII-MDR1, MDCKII-MRP1, MDCKII-MRP2 and MDCKII-MRP3 were generated by stable transfection with the corresponding cDNA into MDCKII cells. Transfected cell lines were provided by Dr P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). The parental cell line MDCKII (available from ATCC, Manassas, VA, USA) was used as a control. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin sulphate.

LLC-PK1 and L-MDR1 cells

L-MDR1 cells, a porcine kidney epithelial cell line overexpressing the human ABCB1 gene, and the corresponding parental cell line LLC-PK1 (available from ATCC, Manassas, VA, USA) as a control were used for ABCB1 inhibition assays. The L-MDR1 cell line was kindly provided by Dr A. H. Schinkel (Netherlands Cancer Institute, Division of Experimental Therapy, Amsterdam, The Netherlands). The cells were cultured under standard cell culture conditions with medium M199 supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin sulphate. To maintain ABCB1 expression the culture medium for L-MDR1 was supplemented with 0.64 μmol/L vincristine. One day before the calcein assay, both cell lines were fed with vincristine-free culture medium.

P388 and P388/dx cells

The murine monocytic leukaemia cell line P388 and the corresponding doxorubicin-resistant cell line P388/dx overexpressing mdr1a/b were used for ABCB1 inhibition assays. Both cell lines were kindly provided by Dr D. Balinari (Pharmacia & Upjohn, Milan, Italy). The cells were cultured under standard cell culture conditions with RPMI 1640 medium containing 10% FCS, 2 mmol/L glutamine, 500 mmol/L β-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin sulphate. To maintain ABCB1 expression, the culture medium for P388/dx was supplemented...
with 0.43 μmol/L doxorubicin. One day before the assay, both cell lines were fed with doxorubicin-free culture medium.

**LS180 cells**

The human colon adenocarcinoma cell line LS180 (available at ATCC, Manassas, VA, USA) was used for induction experiments as a surrogate for the intestine, a major site of drug interactions.\(^{25-27}\) Cells were cultured under standard cell culture conditions with DMEM supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulphate and 0.1 mmol/L non-essential amino acids.

**Cytotoxicity assay**

Maraviroc, vicriviroc, raltegravir and elvitegravir were tested for cytotoxic effects prior to ABCB1, ABCG2 and ABC2C inhibition assays with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany), a colorimetric assay for the quantification of the activity of lactate dehydrogenase, which is released from the cytosol of damaged cells. Cytotoxicity was expressed as a percentage of the effect obtained with the positive control (1% Triton X-100). Inhibition assays were conducted with concentrations that have been shown to be non-toxic in the respective cell line.

**ABCB1 inhibition assay (calcein uptake assay)**

The calcein assay was used to assess ABCB1 inhibition in L-MDR1 cells and P388/dx cells. The assay was conducted and validated as described previously.\(^{23,28}\) Briefly, the assay was performed using 96-well microtitre plates. Adherent L-MDR1 cells were seeded 3 days prior to the experiment. Before the experiment, cells were washed with HBSS supplemented with 10 mmol/L HEPES (HBSS) and preincubated for 30 min at 37°C. After incubation with the test compounds for 10 min, calcein-AM was added for a further 60 min of incubation. Afterwards, uptake was stopped on ice and cells were washed twice with pre-cooled HBSS. Cells were lysed with 1% Triton X-100 for 15 min and calcein fluorescence was measured with 485 nm excitation and 535 nm emission filters in a Fluoroskan Ascent fluorometer (Lab-systems, Frankfurt, Germany). Each concentration was tested in triplicate and each experiment was performed at least in triplicate or in duplicate if no inhibition was seen. For calculation of the inhibitory effects in the calcein assay, the f2 value (concentration needed to increase baseline fluorescence by factor 2) was calculated as described and validated previously\(^{23,29}\) because some compounds did not reach plateau effects.

**ABCG2 inhibition assay (PhA flow cytometry efflux assay)**

The flow cytometric ABCG2 inhibition assay was performed as described and validated previously.\(^{23}\) Briefly, the accumulation of the fluorescent reporter substrate PhA in the ABCG2-overexpressing cell line MDCKII-BCRP was compared with the accumulation in the control cell line MDCKII. Cells (1×10⁶) were incubated for 30 min in RPMI medium supplemented with 2% FCS only as a negative control and in RPMI/2% FCS containing 1 μmol/L PhA. After incubation, cells were washed and subsequently incubated with RPMI/2% FCS as a negative control, with the ABCG2 inhibitor FTC as a positive control at a concentration of 10 μmol/L and with various concentrations of the test compounds. After incubation for 60 min at 37°C on a rotary shaker, cells were washed and resuspended in pre-cooled PBS with 2% FCS. Intracellular PhA fluorescence was analysed in a Becton Dickinson LSR II flow cytometer with a sapphire blue laser and a 530/30 bandpass filter for green fluorescent protein and a 633 nm helium/neon laser and a 660/20 bandpass filter for PhA. In each sample, 30,000 cells were analysed. Living cells were gated using forward versus side scatter. ABCG2-overexpressing MDCKII-BCRP cells were additionally gated by their green fluorescent protein signal. Quantification of inhibition was calculated as published previously.\(^{30}\) Each experiment was performed at least three times on different days.

**ABCC2 inhibition assay**

To assess ABCC2 inhibition we used the MR2 PREDIVE™ Vesicular Transport Kit (SolvBio Biotechnology, Budapest, Hungary). The inhibitory effect of test compounds on ABCC2 was quantified by accumulation of the fluorescent reporter substrate 5(6)-carboxy-2′,7′-dichlorofluorescein (DCF) in inverted vesicular membranes containing ABCC2 transporters. The assay was conducted according to the manufacturer’s instructions.

**Growth inhibition assay**

Many antiretroviral drugs accumulate to toxic intracellular concentrations in cell cultures at high extracellular concentrations.\(^{31}\) ABC transporters are efflux transporters that influence the intracellular concentration of antiretrovirals like PIs. Changes in efflux activity of ABC transporters may alter the intracellular concentration and subsequent sensitivity of cells to antiretrovirals. We therefore used the growth inhibition assay in MDCKII cells overexpressing human ABCB1, ABCG2 and ABC1C-3 as a surrogate for intracellular concentrations and substrate characteristics of the new antiretroviral agents, as this assay has been standardized for cytostatic drugs\(^{32}\) and already described for antiretroviral drugs.\(^{33,34}\) Overexpression of the corresponding transporter leads to decreased intracellular concentrations. This results in higher IC₅₀ values of transported antiretrovirals in transporter-overexpressing MDCKII cells compared with the IC₅₀ values in the MDCKII parental cell line used as a control. Antiretrovirals were tested at 10 concentrations spread over a wide range between 0.005 and 100 μmol/L for maraviroc and elvitegravir and between 0.01 and 500 μmol/L for raltegravir and vicriviroc. Concentration–response curves and IC₅₀ values were calculated by GraphPad Prism version 5.02 (GraphPad Software Inc., La Jolla, CA, USA) according to a sigmoid E₅₀ model. Furthermore, we used the growth inhibition assay in LS180 cells to determine optimum concentrations for the induction assay, as the concentration for induction should not have profound antiproliferative effects. The maximum concentration for induction was set to the IC₅₀ for cell proliferation inhibition.\(^{34}\) Proliferation was quantified by Crystal Violet staining as described previously with minor modifications.\(^{27}\) In brief, a 100 μL aliquot of each cell suspension at a concentration of 1×10⁵ cells/mL for MDCKII, MDCKII-MDR1, MDCKII-BCRP, MDCKII-MRP1, MDCKII-MRP2 and MDCKII-MRP3 cells or a concentration of 3×10⁵ cells/mL for LS180 cells was seeded onto collagen-coated 96-well microtitre plates and incubated for 24 h. The medium was replaced with drug-containing medium and the cells were incubated for a further 48 h. After incubation with test compounds, the cells were washed once with PBS and viable adherent cells were stained with 50 μL of 0.5% Crystal Violet (2.5 g of Crystal Violet in 20% methanol in sterile water) per well for 15 min. After staining, plates were washed with sterile water and dried for 4 h in a drying chamber at 37°C or at room temperature for 12 h. To dissolve the Crystal Violet, 200 μL of methanol was added in each well. Absorption was measured at 555 nm excitation using a Multiskan RC photometer. Proliferation was expressed as the proliferation index, which was calculated as the absorption intensity of the test well in percentage points relative to zero proliferation (absorption of wells containing only medium set to 0%) and native proliferation (absorption intensity of untreated control cells set to 100%). Each experiment was performed at least in triplicate with n=8 wells for each concentration.
Induction assay
LS180 cells were seeded in 75 cm² culture flasks and incubated for 3 days. Cells were then treated with culture medium containing maraviroc (1 or 10 μmol/L), vicriviroc (1 or 10 μmol/L), raltegravir (1 or 10 μmol/L) and elvitegravir, which were dissolved in DMSO, all incubation media, including the positive control and negative control (vehicle control), were supplemented with DMSO to reach an equal concentration of 0.1% DMSO in all solutions. In vicriviroc and raltegravir experiments (aqueous stock solutions) only the positive control (rifampicin) contained 0.01% DMSO, a concentration that has been shown to have no influence.

Quantification of mRNA expression by real-time RT–PCR
RNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). Quality and concentration were measured spectrophotometrically and isolated RNA was stored at −80 °C until analysis. cDNA was synthesized with the Transcripter First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. mRNA expression was quantified by real-time RT–PCR with the LightCycler™ or the LightCycler™ 480 (Roche Applied Science, Mannheim, Germany). PCR amplification was carried out in a 20 μL reaction volume containing 5 μL of 1:10 diluted cDNA, 1× LightCycler-FastStart DNA Master SYBR Green I, 1× LightCycler-FastStart DNA MasterPlus SYBR Green I, 1× LightCycler Taq Man Master (all from Roche Applied Science, Mannheim, Germany) or 1× Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany), 0.3–0.5 μmol/L of each primer and between 3 and 4 mmol/L MgCl₂. Primer sequences are summarized in Table 1. The most suitable housekeeping gene for normalization in LS180 cells was identified using geNorm (version 3.4, Center for Medical Genetics, Ghent, Belgium), which determines the most stable reference genes from a set of tested genes in a given cDNA sample panel. Glucuronidase β (GU) proved to be the most stable housekeeping gene in LS180 cells under our experimental conditions. Data were evaluated by calibrator-normalized relative quantification with efficiency correction using the ReQuant software version 1.01 or LightCycler™ 480 software version 1.5 (Roche Applied Science, Mannheim, Germany) as described previously. The software calculated the relative amount of the target gene and the reference gene (GU) based on the crossing points. Results were expressed as the target/reference ratio divided by the target/reference ratio of the calibrator. The results are therefore corrected for sample inhomogeneities and variance caused by detection. All samples were amplified at least in duplicate.

Functional assay for ABCB1 (rhodamine 123 efflux)
Induction of ABCB1 transport activity in LS180 cells was assessed by the flow cytometric rhodamine 123 assay. In brief, cells (1 × 10⁶/sample) were incubated under light protection with the ABCB1 substrate rhodamine 123 (0.4 μmol/L) followed by an incubation with the specific ABCB1 inhibitor LY335979 (1 μmol/L) or with medium alone at 37 °C on a rotary shaker. Intermittent washing steps were performed at 4 °C with pre-cooled PBS (centrifugation for 5 min at 800 g). Intracellular fluorescence was quantified in an LSRII flow cytometer (Becton Dickinson, Heidelberg, Germany) with FACSDiva Software (Becton Dickinson, Heidelberg, Germany). Living cells were gated using forward versus side scatter. ABCB1 activity was determined as the ratio between the median fluorescence of LY335979 (1 μmol/L)-incubated cells (ABCBI inhibited) and medium-incubated cells (not inhibited) normalized to the corresponding ratio of the negative control in the induction assays (without pretreatment with any drug).

Table 1. Primer sequences

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*Not published yet, primers were used in combination with UPL Probe 38 (Roche Applied Sciences, Mannheim, Germany).
Statistical analysis

Data were analysed using GraphPad Prism Version 5.02 and InStat Version 3.06 (GraphPad Software, San Diego, CA, USA). The differences in mRNA expression and ABCB1 function following incubation with antiretrovirals compared with the respective vehicle controls were tested by analysis of variance (ANOVA) with Dunnett’s post hoc test. Induction was only considered relevant if mRNA expression was at least 1.5-fold base level. Repression was defined as relevant if mRNA levels were decreased less than 0.67-fold base level. Differences in IC50 values of proliferation assays in overexpressing cells compared with the parental cell line MDCKII were also evaluated using ANOVA with Dunnett’s post hoc test. P≤0.05 was considered significant.

Results

ABCB1 inhibition

Maraviroc showed no mdr1a/1b or ABCB1 inhibition up to the tested concentration of 50 µmol/L in P388/dx and 100 µmol/L in L-MDR1 cells and raltegravir also did not inhibit mdr1a/1b or ABCB1 at the maximum tested concentration of 50 µmol/L in P388/dx and 100 µmol/L in L-MDR1 cells. However, vicriviroc and elvitegravir clearly increased calcein fluorescence in P388/dx and L-MDR1 cells, but not in the corresponding parental cell lines, which served as negative controls, indicating inhibition of ABCB1. Figure 1 shows one representative example of a calcein assay with vicriviroc and elvitegravir in P388/dx cells (Figure 1a) and L-MDR1 cells (Figure 1b). The inhibitory potency was expressed as f2 values (Table 2).

ABCG2 inhibition

In the ABCG2 inhibition assay elvitegravir and vicriviroc increased PhA fluorescence in MDCKII-BCRP cells (Figure 2). Vicriviroc did not reach maximum effects up to the highest concentration tested (500 µmol/L). Therefore, the IC50 value was estimated based on the maximum effect observed with the positive control FTC. Maraviroc and raltegravir increased PhA fluorescence in MDCKII-BCRP cells only minimally (data not shown), indicating only very weak ABCG2 inhibition. The data are summarized in Table 3.

ABCC2 inhibition

The positive control MK571 reduced the transport of the fluorescent dye CDCF into the inside-out membrane vesicles overexpressing human ABCC2 with an IC50 of 6.6±0.1 µmol/L (Figure 3). Maraviroc and vicriviroc, however, increased fluorescence, indicating increased transport of CDCF into the vesicles (Figure 3). Raltegravir had no effects at all and for elvitegravir also no substantial effects were observed. Autofluorescence of the test compounds was not detected.

Proliferation assay

Proliferation assays in MDCKII cells and MDCKII cells overexpressing ABCB1, ABCG2, ABCC1, ABCC2 or ABCC3 were conducted to detect substrate characteristics. Significant differences in the resistance of MDCKII and MDCKII-MDR1 cells indicated that elvitegravir and vicriviroc are ABCB1 substrates (Table 4).

Table 2. f2 values for mdr1a/b and ABCB1 inhibition in P388/dx and L-MDR1 cells

<table>
<thead>
<tr>
<th>Test</th>
<th>f2 value in P388/dx (µmol/L)</th>
<th>f2 value in L-MDR1 (µmol/L)</th>
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<tr>
<td>Elvitegravir</td>
<td>1.9±0.2</td>
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<td>ND</td>
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<tr>
<td>Vicriviroc</td>
<td>8.5±3.6</td>
<td>31.9±13.9</td>
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<td>Quinidine</td>
<td>2.4±0.918</td>
<td>13.2±3.829</td>
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</table>

ND, not determinable. Data are expressed as means ± SD.

Figure 1. mdr1a/b and ABCB1 inhibition measured by calcein assay. Concentration-dependent effects of elvitegravir and vicriviroc on calcein accumulation in P388/dx cells (a) and L-MDR1 cells (b). Each curve depicts one representative experiment of a series of three or four. Data are expressed as means ± SEM.
Inhibition of BCRP/ABCG2 by elvitegravir and vicriviroc. Each curve depicts one representative experiment of a series of three or four; each concentration was tested in 30,000 cells.

**Table 3.** IC\(_{50}\) values for ABCG2 inhibition in MDCKII-BCRP cells

<table>
<thead>
<tr>
<th>Test compound</th>
<th>IC(_{50}) ((\mu)mol/L)</th>
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<tbody>
<tr>
<td>Elvitegravir</td>
<td>15.7 ± 5.7</td>
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<tr>
<td>Maraviroc</td>
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<td>Raltegravir</td>
<td>ND</td>
</tr>
<tr>
<td>Vicriviroc</td>
<td>236.7 ± 93.3(^a)</td>
</tr>
<tr>
<td>FTC</td>
<td>0.7 ± 0.3(^{61})</td>
</tr>
</tbody>
</table>

ND, not determinable.

Data are expressed as means ± SD.

\(^a\)IC\(_{50}\) is estimated (with maximum set to positive control FTC) because plateau effects are not reached due to toxic effects of solvent.

MDCKII-BCRP and MDCKII-MRP1-3 cells was inhibited by about 40% by raltegravir, but not in MDCKII-MDR1 cells, indicating that raltegravir is transported by ABCB1.

**Induction**

Proliferation assays in LS180 cells were conducted prior to induction assays for determination of suitable concentrations of antiretrovirals to minimize antiproliferative effects during the induction period. Results demonstrated that elvitegravir inhibited proliferation of LS180 cells with an IC\(_{20}\) of 8.4 ± 0.9 \(\mu\)mol/L. Therefore, induction was carried out with a maximum concentration of 10 \(\mu\)mol/L. Due to low toxicities of maraviroc, raltegravir and vicriviroc, showing only marginal cell proliferation inhibition at concentrations above 50 \(\mu\)mol/L, calculation of IC\(_{20}\) values was impossible. For adequate comparability, these antiretrovirals were also tested at 1 and 10 \(\mu\)mol/L.

Induction of mRNA expression of drug transporters, PXR and relevant CYP enzymes in LS180 cells was analysed after 4 days of treatment with antiretrovirals. ABCB1 mRNA was significantly and strongly induced by elvitegravir at 1 and 10 \(\mu\)mol/L (Figure 4). ABCC1 was only weakly induced by raltegravir (1.8 ± 0.7 at 1 \(\mu\)mol/L and 2.0 ± 0.6 at 10 \(\mu\)mol/L). ABCBC3 was slightly induced by elvitegravir (1.9 ± 0.5 at 1 \(\mu\)mol/L, P < 0.05, and 2.0 ± 1.0 at 10 \(\mu\)mol/L, P < 0.05) and maraviroc (2.3 ± 0.8 at 10 \(\mu\)mol/L, P < 0.01).

The mRNA expression of ABCC2 and ABCC4 was not influenced by any compound tested. ABCC2 was weakly induced by elvitegravir (1.6 ± 0.4 at 10 \(\mu\)mol/L, P < 0.05) and maraviroc (1.7 ± 0.4 at 1 \(\mu\)mol/L, P < 0.05).

CYP3A4 mRNA expression was strongly induced by 10 \(\mu\)mol/L elvitegravir (Figure 5). None of the compounds tested induced
Figure 4. Effect of antiretrovirals and positive control rifampicin (10 μmol/L) on ABCB1 mRNA expression in LS180 cells compared with medium control. Expression data were normalized to reference gene GU. Data are expressed as means ± SEM for n = 8–12 (four biological replicates and two or three PCR runs for every sample). *P < 0.05, **P < 0.01.

Figure 5. Effect of antiretrovirals and positive control rifampicin (10 μmol/L) on CYP3A4 mRNA expression in LS180 cells compared with medium control. Expression data were normalized to reference gene GU. Data are expressed as means ± SEM for n = 8–12 (four biological replicates and two or three PCR runs for every sample). **P < 0.01.

mRNA of PXR or CYP3A5 whereas CYP2B6 mRNA was significantly induced by elvitegravir (1.7 ± 0.6 at 10 μmol/L, P < 0.05). Among the SLCOs tested, only SLCO1B1 was significantly induced by 10 μmol/L elvitegravir (1.7 ± 0.3 at 10 μmol/L, P < 0.01).

For compounds with significant induction of ABCB1 mRNA expression at 10 μmol/L, induction was also quantified at the functional level after 3 days and 1 week of incubation. After 3 days of treatment, a trend towards increased ABCB1 activity was observed for rifampicin and elvitegravir (Figure 6). After 7 days ABCB1 activity was significantly increased by elvitegravir, vicriviroc and the positive control, rifampicin (Figure 6).

Discussion

Effective treatment of HIV-1 infections generally requires a combination of NNRTIs, NRTIs, PIs or the novel CCR5 antagonists and integrase inhibitors. Drug–drug interactions among antiretrovirals are frequent and are primarily mediated by modulation of activity and expression of CYPs, efflux and uptake transporters, leading to subtherapeutic or toxic concentrations, or the formation of sanctuary sites with reduced drug access.57 Drug interactions may thus compromise the safety and effectiveness of HAART. However, data on the interaction potential of the CCR5 antagonists maraviroc and vicriviroc and the integrase inhibitors elvitegravir and raltegravir are sparse. We therefore investigated the interaction of these drugs with key transporters for HAART. Table 5 summarizes the most important findings.

Maraviroc is a known high-affinity ABCB1 substrate.12–14 Our study used proliferation assays for investigation of substrate characteristics. Although it has been demonstrated that proliferation assays are a suitable tool to detect substrates,27,32,33 the low toxicity of maraviroc rendered it impossible to evaluate whether it is transported by ABCB1, ABCC1-3 or ABCG2. However, we verified that maraviroc is not an inhibitor of ABCB1 or ABCG2. Interestingly, maraviroc seems to stimulate ABCB2 transport. In contrast to the control inhibitor MK571, which decreased CDCF fluorescence in the inside-out vesicles, maraviroc increased fluorescence in a concentration-dependent manner. Autofluorescence as an underlying reason could also be ruled out, as unspecific access of CDCF to vesicles as fluoroscence did not increase in the absence of ATP. It is thus possible that maraviroc activated ABCB2, as has been described for other drugs.16,35 A possible mechanism for the activation might be the existence of several binding sites of ABCB2, as proposed by Zelcer.
Table 5. Interaction of new antiretrovirals with drug transporters (including induction of CYP3A4 and CYP2B6)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Maraviroc</th>
<th>Vicriviroc</th>
<th>Raltegravir</th>
<th>Elvitegravir</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>ABCB1</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ABCB2</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB1</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>ABCB2</td>
<td>activator</td>
<td>activator</td>
<td>–</td>
<td>ambiguous</td>
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<tr>
<td>ABCB2</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Inductor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>SLC01B1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

ND, not determinable due to absence of antiproliferative effects; –, no effect; +, substrate or weak inhibitor/inductor; ++, moderate inhibitor/inductor; ++++, strong inhibitor/inductor.

*aAs demonstrated by studies published previously.12-14 et al.40 If several binding sites are involved, activation can be explained by allosteric binding of the test compound to a regulatory site.

Clinically at least as important as the substrate or inhibitor properties of drugs are enzyme- and transporter-inducing characteristics, which may substantially increase the dose requirements of co-administered drugs and may result in non-response. Maraviroc did not induce CYP3A4, CYP3A5, CYP2B6 or most of the drug transporters tested. In contrast, maraviroc slightly induced ABCB3 and ABCG2 mRNA.

Taken together, these results indicate that the potential of maraviroc to influence the pharmacokinetics of co-administered drugs appears to be low as the effects observed in vitro were not very pronounced. So far, there are no clinical data corroborating the inhibitory or inducing properties of maraviroc, but no studies have been conducted that directly addressed this issue. However, we cannot rule out that ABCB2 activation leads to modulated distribution of ABCB2 substrates and that the induction of some of the transporters investigated might also modulate the disposition of other drugs.

The CCR5 inhibitor vicriviroc is still under development and its potential for interactions has not been investigated so far. Our study indicated for the first time that vicriviroc is an ABCB1 substrate, but not a substrate of ABCG2 or ABCC1-3. Moreover, our study demonstrated that vicriviroc inhibits ABCB1 with a potency similar to amprenavir and saquinavir (Table 2) and also stimulates ABCB2 activity, as demonstrated for maraviroc (Figure 3). Plasma C_{max} levels for vicriviroc have been reported to be in the range of 0.28 μmol/L on day 14 of vicriviroc 25 mg twice daily. Plasma levels therefore are expected to be lower than concentrations necessary for ABCB1 inhibition or ABCC2 stimulation. The local concentration in the intestine, however, is probably substantially higher during dissolution of the formulation and subsequent drug absorption. As the intestine is a major site of drug–drug interactions mediated by ABCB1, the pharmacokinetics of substrates of ABCB1 and possibly also of ABCC2 could be modulated. In contrast, the potential of vicriviroc to inhibit ABCG2 is most likely too weak to be clinically relevant. An induction assay showed that vicriviroc significantly increased ABCB1 function after 7 days of treatment (Figure 6), though the effect was not significant after 3 days at the mRNA level (Figure 4). Moreover, vicriviroc did not induce the other tested transporters and CYP enzymes. The clinical relevance of ABCB1 induction is questionable, because vicriviroc has also been revealed to be an inhibitor of this transporter. Taking these results together, the interaction potential of vicriviroc at the level of drug transporters is presumably low, but so far clinical studies verifying this assumption are missing.

The results of the proliferation assays indicate that raltegravir is only transported by ABCB1. Concurrently, our results demonstrate that raltegravir does not inhibit ABCB1, which is in line with previous in vitro results. Moreover, it does not inhibit ABCG2 or ABCC2. In the induction assay only ABCB1 expression was significantly induced by raltegravir (1.7-fold at 1 μmol/L and 2-fold at 10 μmol/L). C_{max} plasma levels have been reported to be in the range of 1.3–5.3 μmol/L, possibly resulting in potentially clinically relevant induction of ABCB1, at least in the intestine. Moreover, our data corroborate previously published data that raltegravir does not induce CYPs. In summary, raltegravir appears to be the drug that is least prone to drug–drug interactions among the antiretrovirals tested in this study.

In contrast, the integrase inhibitor elvitegravir, which has not been approved yet, reveals, at least in vitro, a substantial interaction potential. Our data indicate that elvitegravir is transported by ABCB1 but not by the other ABC transporters tested. When co-administered with atazanavir/ritonavir or lopinavir/ritonavir, the area under the curve of elvitegravir was increased about 2-fold, which might have been caused not only by CYP3A4 and UGT1A inhibition but also, at least partly, by ABCB1 inhibition.

Moreover, compared with the well-known and strong ABCB1 inhibitor quinidine, elvitegravir is an equipotent inhibitor (Table 2) and it also inhibits ABCG2 (Table 3). Furthermore, our results demonstrate strong inducing properties for elvitegravir. It induced many transporters and all CYPs tested and for CYP3A4 and ABCB1 it was equipotent to the positive control, rifampicin, suggesting that it might cause clinically relevant drug–drug interactions with CYP3A4 or ABCB1 substrates. For ABCB1 we also confirmed induction at the functional level. In contrast to the mRNA induction, the increase in ABCB1 function needs a longer time period to become significant (Figure 6), because mRNA has to be translated into the protein and the latter has to be processed and integrated into the membrane before an effect becomes visible. Clinical data on drug–drug interactions with elvitegravir are sparse and there is no published study investigating the effect of long-term application of elvitegravir on the pharmacokinetics of CYP3A4 or ABCB1 substrates. Thus, no in vivo data exist corroborating the strong inducing effects observed in vitro. However, elvitegravir also revealed
strong inhibitory effects, which might be superimposed on induc-
tion. This might be the reason why elvitegravir has so far been
reported to have a modest potential to be involved in clinically
meaningful drug interactions.\(^5\)\(^6\) However, there is one study
demonstrating that the \(C_{\text{max}}\) of the ABCB1 substrate maraviroc
increased by factor 2.2 when co-administered with ritonavir-
boosted elvitegravir.\(^1\)\(^4\) The authors hypothesized inhibition of
CYP3A4 and ABCB1 by ritonavir, but inhibition of ABCB1 by elvite-
gravir also appears possible. Effective concentrations for ABCB1
inhibition in vitro match the reported \(C_{\text{max}}\) levels found in clinical
trials (1–2.1 \(\mu\)mol/L depending on dosing scheme).\(^4\)\(^6\) Thus,
drug–drug interactions of elvitegravir with ABCB1 substrates
could possibly be clinically relevant and require further attention
in in vivo studies.

Some limitations of this study merit mentioning. We have
analysed a selection of drug transporters and CYPs known to
contribute to drug–drug interactions of antiretrovirals. Other
transporters might also modulate the safety and effectiveness
of therapy with these novel antiretrovirals. Furthermore, for all
enzymes and transporters except ABCB1 we have quantified
induction at the level of mRNA expression. Increased mRNA
expression does not necessarily result in an augmented protein
or activity level. It has, however, been shown that changes in
mRNA expression are in the majority of cases associated with
changes in the corresponding protein level\(^4\)\(^7\)\(^–\)\(^4\)\(^9\) or altered function.

Generally, LS180 cells as an induction model are
most suitable for the investigation of induction mediated by
the PXR. Because constitutive androstane receptor 1 (CAR1),
which is expressed in hepatocytes, is apparently not expressed
in LS180 cells,\(^2\)\(^6\) it is a weak model for CAR1-mediated induction
of transporters and CYPs, such as P-gp.\(^5\)\(^0\)

This study set out to determine the potential for drug–drug
interactions of novel antiretrovirals. The evidence from this
study suggests that clinically relevant drug–drug interactions
of these novel antiretrovirals, especially elvitegravir, might
occur, because elvitegravir modulates the expression of
CYP3A4, ABCB1 and ABCG2 and the function of ABCB1 and
ABCG2. At this point in time, data on drug interactions are
sparse and it is therefore difficult to define those interactions
that affect the safety and effectiveness of therapy. However,
given the strong interaction potential of elvitegravir observed
in vitro, our data should guide the design of drug–drug inter-
actions studies with this compound in vivo.

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Transparency declarations

None to declare.

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