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Comparison of the inhibitory activity of anti-HIV drugs on P-glycoprotein

Caroline Henrike Storch, Dirk Theile, Heike Lindenmaier, Walter Emil Haefeli, Johanna Weiss *

Department of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Im Neuenheimer Feld 410, D-69120 Heidelberg, Germany

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ABSTRACT

Human immunodeficiency virus 1 (HIV-1) infections are treated with HIV-protease inhibitors (PIs), nucleoside (NRTIs), non-nucleoside (NNRTIs), and nucleotide reverse transcriptase inhibitors (NtRTIs). The combined administration of antiretrovirals improves patient outcomes while increasing the likelihood of drug interactions. Indeed, as substrates, inhibitors, and occasionally also inducers of P-glycoprotein (P-gp) PIs may substantially alter the pharmacokinetics of co-administered drugs. However, the P-gp inhibitory potencies specified in the numerous publications are not comparable, because they were determined with different assays and cell lines. Moreover, data on the interaction of other anti-HIV drugs with P-gp are sparse and conflicting. We therefore aimed to clarify, which anti-HIV drugs inhibit P-gp and to compare the inhibitory potencies using two independent standard methods (calcein uptake assay, flow cytometric rhodamine123 efflux assay). In the calcein assay, all PIs, all NNRTIs, abacavir, and tenofovir disoproxil fumarate acted as P-gp inhibitors with largely differing potencies between compounds. In P388/dx cells the ranking order of inhibition was: nelfinavir > ritonavir > tipranavir > lopinavir > quinidine (positive control) > delavirdine > saquinavir > amprenavir > atazanavir > efavirenz > nevirapine > abacavir > tenofovir disoproxil fumarate. In conclusion this is the first study to provide comprehensive information on the P-gp interaction profile of anti-HIV drugs under identical assay conditions. Our study reveals that many compounds may indeed inhibit P-gp substantially and further indicates that of the various systems tested, the calcein assay in P388/dx/P388 cells is the most suitable and reliable in vitro model for the quantification of P-gp inhibition.

* *Corresponding author*. Tel.: +49 6221 56 39402; fax: +49 6221 56 4642.
E-mail address: johanna.weiss@med.uni-heidelberg.de (J. Weiss).

Abbreviations: BCRP, breast cancer resistance protein; CYP, cytochrome P450 isozyme; *f2*, concentration needed to increase baseline fluorescence by factor 2; FCS, foetal calf serum; FTC, fumitremorgin C; HAART, highly active antiretroviral therapy; LY335979, zosuquidar; HBSS, Hanks' balanced salt solution; HHBSS, Hanks' balanced salt solution with 1% HEPES; HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus 1; PI, HIV protease inhibitor; MF, median fluorescence; MRP, multidrug resistance associated protein; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; pBCECs, porcine brain capillary endothelial cells; P-gp, P-glycoprotein; S.E.M., standard error of the mean; tenofovir DF, tenofovir disoproxil fumarate

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1. Introduction

Five different classes of anti-HIV drugs are currently used to treat patients with human immunodeficiency virus 1 (HIV-1) infections. They consist of HIV-protease inhibitors (PIs), nucleoside (NRTIs), non-nucleoside (NNRTIs), and nucleotide reverse transcriptase inhibitors (NtRTIs), and newly also HIV-fusion inhibitors. The combined administration of three or four antiretrovirals as highly active antiretroviral therapy (HAART) substantially improves patient outcomes while increasing the likelihood of drug interactions [1,2]. NNRTIs and PIs have a particular disposition to generate drug interactions, predominantly as a result of their ability to inhibit or induce drug metabolising cytochrome P450 isozymes (CYPs) [1,2]. Such interactions may be favourable and indeed enzyme inhibition by the PI ritonavir is even considered critical to boost PI bioavailability and produce effective concentrations [3]. Beyond CYPs, drug transporters like P-glycoprotein (P-gp, MDR1/ABCB1) represent a common target for drug-interactions with anti-HIV drugs [1,4–6].

P-gp is the best-investigated member of the ATP-binding cassette superfamily of membrane transport proteins. It is localised in the apical membrane of many epithelial and endothelial cells and participates in absorption and excretion of drugs. Moreover, as an active membrane-bound transporter it may also form barriers for drug distribution like the blood–brain barrier, and may thus critically modulate the pharmacokinetics of substrates of this efflux pump [7] through drug–drug interactions [8].

Indeed, as substrates, inhibitors, and occasionally also inducers of P-gp [9–15] PIs may influence the pharmacokinetics of concomitantly administered drugs in multiple ways [1,16,17]. However, the P-gp inhibitory potencies specified in the numerous independent publications are hardly comparable, because they were determined with different assays in different cell lines. Consequently, extrapolation of the clinical relevance of such findings and definition of the extent of interaction relative to other drugs is almost impossible. Therefore, it is still debatable, which PIs are the most potent P-gp inhibitors and for the possible inhibition of P-gp by tipranavir no information is yet available.

The NNRTIs delavirdine, efavirenz, and nevirapine reportedly have P-gp-inducing properties [18,19], but results assessing whether they are also substrates and/or inhibitors of P-gp are conflicting [1,5,18]. Also on the potential interaction of NRTIs, tenofovir, and its prodrug tenofovir disoproxil fumarate (tenofovir DF) with P-gp only limited information is available [20–25]. Moreover, the few studies used different methods leading to restricted comparability and ambiguous conclusions.

Therefore, the objectives of this study were: (1) to clarify, whether tipranavir (and other PIs), NNRTIs, NRTIs, and tenofovir inhibit P-gp and (2) to compare the inhibitory potencies of all these compounds using two independent standard methods for the evaluation of P-gp inhibition (calcein assay and rhodamine123 assay). As models for human, murine, and porcine P-gp three different cell lines (L-MDR1, P388/dx, and primary cell cultures of porcine brain capillary endothelial cells (pBCECs)) were used in the calcein assay, in the rhodamine123 assay only P388/dx and the corresponding parental cell line were used.

2. Materials and methods

2.1. Compounds

Media, foetal calf serum (FCS), medium supplements, antibiotics, glutamine, HEPES, and Hanks' balanced salt solution (HBSS) were purchased from Invitrogen (Karlsruhe, Germany), DMSO was from AppliChem. Calcein-acetoxymethyl ester was purchased from MobiTec (Göttingen, Germany), rhodamine123 from Calbiochem (San Diego, USA), MK571 from BIOMOL Research Laboratories (Plymouth, USA), probenecid from Sigma Aldrich (Taufkirchen, Germany), and the Cytotoxicity Detection Kit was from Roche Applied Science (Mannheim, Germany). FTC was kindly provided by the National Cancer Institute (Rockville, USA) and LY335979 (zosuquidar) by Eli Lilly Company (Bad Homburg, Germany). Anti-HIV drugs were obtained from Sigma–Aldrich (Taufkirchen, Germany) or from the corresponding manufacturer.

2.2. Stock solutions and test solutions

Stock solutions of test compounds were prepared strictly following the manufacturers instructions. Only very few compounds were soluble in aqua bidest. All others were dissolved in dimethyl sulfoxide (DMSO). The DMSO concentration in the assays never exceeded 1% (v/v), a concentration which was found not to influence the results of the assays. The concentration ranges of control compounds and antiretroviral agents used in the assays are listed in Table 1. All anti-HIV drugs were tested up to the highest concentration soluble in aqueous solution and, if soluble, up to 1 mM. If plateau effects were reached the concentrations were not further increased. In the calcein assay 10 different concentrations and in the rhodamine123 efflux assay 8 different concentrations were analysed.

2.3. Cells

As a model for human P-gp we used L-MDR1 cells, a cell line generated by transfection of the porcine kidney epithelial cell line LLC-PK1 with the human MDR1 gene [26] and the parental cell line LLC-PK1 (available at ATCC, Manassas, USA) as a control. The L-MDR1 cell line was kindly provided by Dr. A.H. Schinkel (Amsterdam, The Netherlands). The cells were cultured and seeded as described previously [27].

As a model for murine P-gp we used the murine monocytic leukaemia cell line P388 and the corresponding doxorubicin-resistant cell line P388/dx over-expressing *mdr1a/1b* [28]. Both cell lines were kindly provided by Dr. Dario Ballinari (Pharmacia & Upjohn, Milano, Italy). The cells were cultured and seeded as described previously [29].

As a model for porcine P-gp we used porcine primary brain capillary endothelial cells (pBCECs), which were isolated and cultured as described previously [27].

2.4. Cytotoxicity assay

Each test compound was screened for possible cytotoxic effects with the Cytotoxicity Detection Kit, a colorimetric assay for the quantification of lactate dehydrogenase activity

Table 1 – Tested concentration ranges of controls and antiretroviral drugs

Compound	Tested concentration range (μM)
Controls	
LY335979	0.0005–1
Quinidine	0.005–200
Probenecid	0.05–500
MK571	0.005–100
FTC	0.0005–20
NRTIs/NtRTIs	
Abacavir	0.01–500
Didanosine	0.025–500
Emtricitabine	0.01–1000
Lamivudine	0.01–1000
Stavudine	0.025–1000
Tenofovir	0.0025–1000
Tenofovir-DF	0.1–1000
Zalcitabine	0.01–1000
Zidovudine	0.01–1000
NNRTIs	
Delavirdine	0.0025–100
Efavirenz	0.001–100
Nevirapine	0.001–1000
PIs	
Amprenavir	0.01–100
Atazanavir	0.0005–100
Indinavir	0.0005–20
Lopinavir	0.0005–20
Nelfinavir	0.0005–20
Ritonavir	0.0001–5
Saquinavir	0.001–50
Tipranavir	0.0005–20

released from the cytosol of damaged cells into the supernatant. Cytotoxicity was calculated as a percentage of the effect obtained with the positive control (total cell lysis).

2.5. Quenching test

To exclude that quenching effects might bias the results of the assays, the influence of the test compounds on the fluorescence of the P-gp probe substrates was evaluated by adding increasing concentrations of the test compounds to rhodamine123 solution or to cell lysates after incubation with calcein-AM.

2.6. Calcein uptake assay

The calcein assay was used to assess P-gp inhibition in L-MDR1 cells, P388/dx cells, and pBCECs. The assay was conducted and validated as described previously [27,29]. Each experiment was performed at least in duplicate (if no inhibition was observed) or in triplicate on different days.

2.7. Flow cytometry (rhodamine123 efflux)

The flow cytometric assessment of P-gp inhibition was performed according to [30] with minor modifications. As cell models P388/dx/P388 and L-MDR1/LLC-PK1 systems were used. 10^6 cells were suspended in 500 μl incubation medium (RPMI with 2% FCS) containing rhodamine123 (0.5 μM for P388/dx and P388 cells, 1.5 μM for L-MDR1 and LLC-PK1 cells) and incubated at 37 °C for 30 min on a rotary shaker (450 rpm). Cells were then washed once with 1 ml ice-cold incubation medium and resuspended in 500 μl incubation medium containing the test compounds at various concentrations. After incubation for 50 min at 37 °C on a rotary shaker, cells were washed with 1 ml ice-cold PBS with 2% FCS, resuspended in ice-cold PBS with 2% FCS and kept on ice until flow cytometry. Intracellular fluorescence was analysed in a Becton Dickinson LSR II flow cytometer (Heidelberg, Germany) with a solid state coherent sapphire blue laser and a 530 bandpass filter.

In each sample 30,000 cells were counted. Cell debris was eliminated by gating the living cells in the forward versus side scatter. To quantify the inhibitory effects of the compounds, the ratio between the median fluorescence (MF) with inhibitor and without inhibitor during the efflux period was calculated and normalised to the effect observed in the corresponding parental cell line according to the following equation (Eq. (1)):

$$\text{Inhibition ratio} = \frac{\mathrm{MF}_{\text{overexpressing cell line with test compound}} / \mathrm{MF}_{\text{overexpressing cell line without test compound}}}{\mathrm{MF}_{\text{parental cell line with test compound}} / \mathrm{MF}_{\text{parental cell line without test compound}}} \quad (1)$$

Each experiment was performed at least in triplicate on different days. To exclude possible influences of breast cancer resistance protein (BCRP), multidrug resistance associated proteins (MRPs), or organic anion transporters on the assay, the selective BCRP inhibitor FTC, the selective MRP inhibitor MK571, and the non-selective inhibitor of organic anion transporters and MRPs probenecid were also tested.

2.8. Statistical analysis

For calculation of the inhibitor effects in the calcein assay, the *f*2-value (concentration needed to increase baseline fluorescence by factor 2 ± standard deviation) was calculated as described and validated previously [27,31], because most compounds did not reach plateau effects. For calculation of the IC_{50}-values the following formula (Eq. (2)) was used:

$$y = \frac{I_{max} - I_{min}}{1 + (x/IC_{50})^s} + I_{min} \quad (2)$$

where I_{min} (background) was set to 1 (no inhibition) and I_{max} the maximal inhibition and *s* is the slope factor.

p-Values for the concentration-dependent changes in intracellular rhodamine123 fluorescence were determined by repeated measures ANOVA with post Dunnett's multiple comparison test for posthoc pairwise comparison with the control results obtained without inhibitor. The correlations between *f*2-values in pBCECs or L-MDR1 cells and P388/dx were assessed by Spearman rank correlation and characterised by the corresponding correlation coefficient r_S (GraphPad Prism, version 4.0; GraphPad Software Inc., San Diego, CA). A p-value < 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of the compounds tested

Of all PIs and NNRTIs tested only saquinavir (≥10 μM in the P388/P388/dx system) and efavirenz (>50 μM in all cell systems) revealed cytotoxic effects > 50%. The corresponding values were excluded from the analysis. For the NRTIs no cytotoxic effects were found up to the highest concentration tested except for emtricitabine which had moderate cytotoxic effects at ≥50 μM (<40%).

3.2. Calcein assay

In the calcein assay, all PIs and all NNRTIs acted as P-gp inhibitors with largely differing potencies between compounds (Table 2). Nelfinavir, lopinavir, tipranavir, and ritonavir were the most potent P-gp inhibitors being as or even more potent than the well-known inhibitor quinidine. Fig. 1 depicts the effects of all compounds with inhibitory effects in P388/dx cells. Among the NNRTIs delavirdine exhibited the highest inhibitory potency, followed by a moderate inhibition by efavirenz and only weak inhibition by nevirapine.

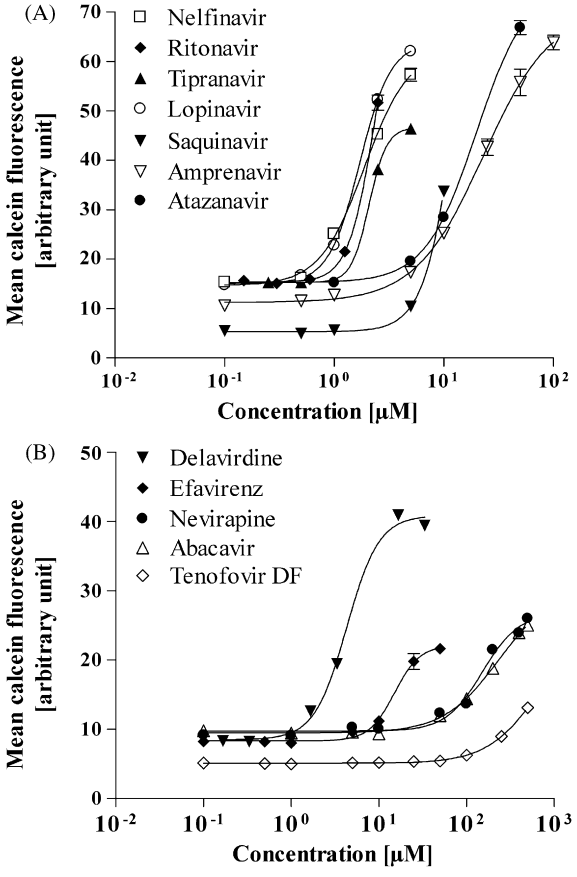

Fig. 1 – Calcein assay: concentration dependent effect of PIs (A) and NNRTIs, abacavir, and tenofovir DF (B) on baseline calcein fluorescence in P388/dx cells. For each cell line one representative experiment of a series of 3–5 is shown. Data are expressed as mean ± S.E.M. for $n = 8$ wells. Only the results of concentrations ≥ 10^{-1} μM are shown.

Table 2 – Inhibition of porcine, murine, and human P-gp (*f*2-values) by PIs, NNRTIs, abacavir, and tenofovir DF in the calcein assay in three different cell lines

Compound	pBCECs	*f*2-Value (μM)	
		P388/dx	L-MDR1
LY335979	0.003 ± 0.0007[a]	0.01 ± 0.006[a]	0.02 ± 0.01[a]
Quinidine	1.12 ± 0.4[a]	2.43 ± 0.9[a]	13.2 ± 3.8[a]
Amprenavir	8.61 ± 0.8[a]	9.01 ± 0.5[a]	N.D.[b]
Atazanavir	3.15 ± 0.7	12.1 ± 3.9	N.D.[b]
Indinavir	16.7 ± 3.5	N.D.[c]	N.D.[c]
Lopinavir	0.69 ± 0.1	2.05 ± 0.2[a]	8.43 ± 1.3
Nelfinavir	0.54 ± 0.2	1.11 ± 0.3[a]	4.99 ± 1.4
Ritonavir	1.29 ± 0.1	1.30 ± 0.0	N.D.[b]
Saquinavir	3.09 ± 0.3	5.08 ± 2.3	27.9 ± 4.4
Tipranavir	0.98 ± 0.3	1.53 ± 0.2	N.D.[b]
Delavirdine	3.01 ± 0.3	3.32 ± 0.2	N.D.[b]
Efavirenz	10.7 ± 2.2	27.2 ± 8.2	25.5 ± 1.9
Nevirapine	120 ± 7.9	133 ± 50	N.D.[c]
Abacavir	278 ± 52	158 ± 8.4	N.D.[c]
Tenofovir DF	N.T.	553 ± 49	N.D.

Data are expressed as mean ± S.D.; N.D., not definable, N.T., not tested.
[a] *f*2-Value previously generated by our research group under identical assay conditions and published earlier [29,31,56].
[b] Correct calculation of the *f*2-value was not possible due to the effect seen in the control cell line LLC-PK1.
[c] *f*2-Value beyond solubility limit.

Abacavir was the only NRTI tested that weakly inhibited P-gp in pBCECs and P388/dx cells (Table 2). There was a small inhibitor effect also in the L-MDR1 cells, but it was too weak to allow calculation of an *f*2-value. The *f*2-values obtained in P388 cells were closely correlated with those in pBCECs (Fig. 2; $r_S = 0.96$, $p < 0.0001$) and in L-MDR1 cells ($r_S = 0.94$, $p = 0.017$).

Whereas none of the compounds tested increased calcein fluorescence in the control cell line P388, indicating specific P-gp inhibition in this cell system, amprenavir, atazanavir, ritonavir, tipranavir, and delavirdine also showed a slight effect in the LLC-PK1 cell system suggesting the involvement of another transporter or of unspecific effects thus precluding calculation of correct *f*2-values. None of the test compounds quenched calcein fluorescence.

3.3. Flow cytometry assay

To validate the flow cytometry assay and to guide the selection of the most suitable cell system, the effect of specific inhibitors of other important active drug transporters on rhodamine123 efflux was evaluated. LY335979 and quinidine as selective P-gp inhibitors (positive control), FTC as a selective BCRP inhibitor, MK571 as an inhibitor of MRPs, and probenecid as a non-selective inhibitor of organic anion transporters and of MRPs were tested in P388/dx, P388, L-MDR1, and LLC-PK1 cells.

LY335979 and quinidine significantly increased intracellular rhodamine123 fluorescence in the respective P-gp over-expressing cell lines and the relative increase was larger in P388/dx than in L-MDR1 cells (Fig. 3). Both inhibitors had no effect in the control cell line P388, but slight effects in LLC-PK1 cells (data not shown) indicating a possible influence on porcine pgp expressed by LLC-PK1. Therefore and due to the higher sensitivity of the P388/dx/P388 system compared to the

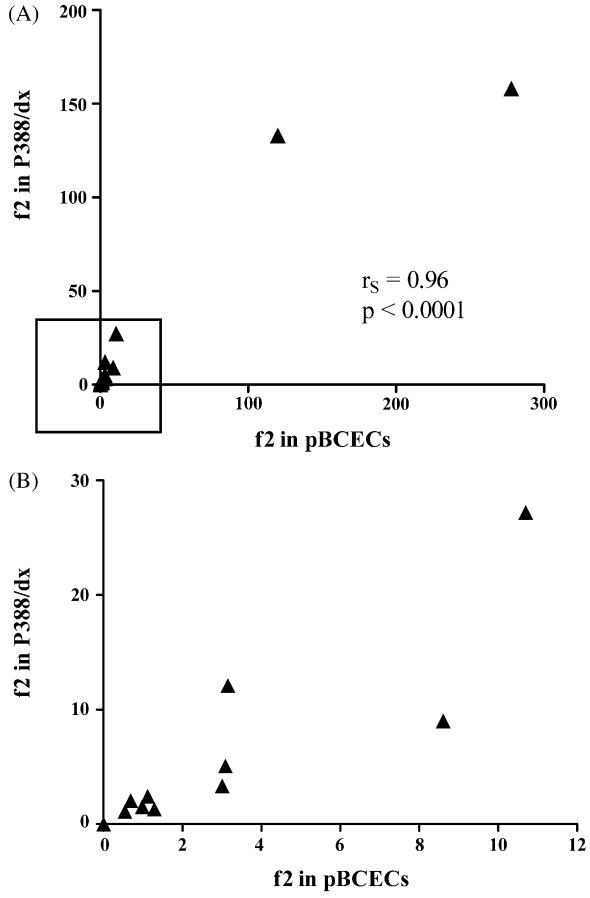

Fig. 2 – Calcein assay: correlation of the ranking order of *f*2-values in pBCECs and in P388/dx cells for the 13 compounds of this study, where calculation of *f*2-value was possible in both cell lines. (B) Represents a zoomed section of (A). Correlation was assessed by Spearman rank correlation. r_S = Spearman rank correlation coefficient.

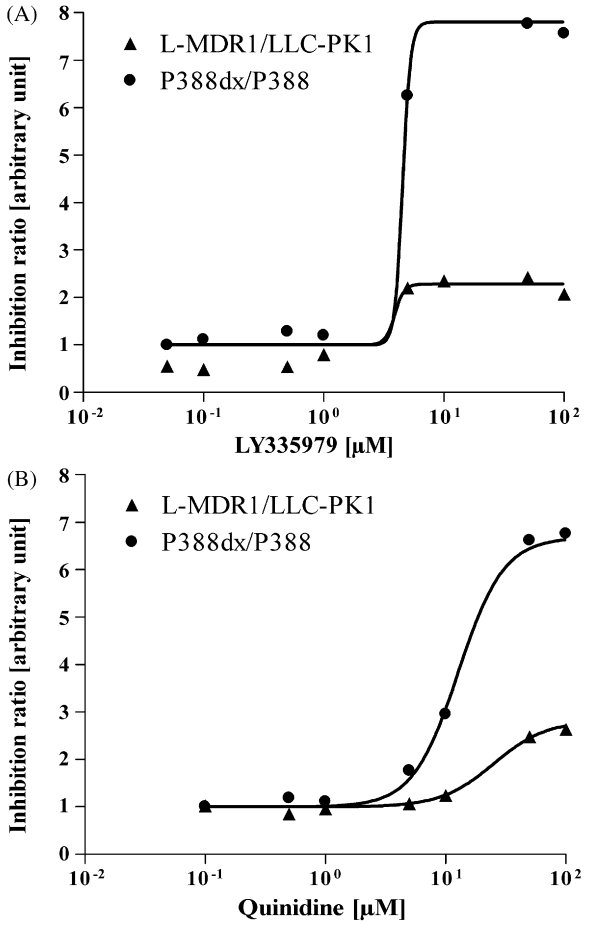

Fig. 3 – Flow cytometry assays: comparison of the inhibition ratio in the L-MDR1/LLC-PK1 and in the P388/dx/P388 cell system demonstrating a higher ratio in the latter. Data are shown as inhibition ratio according to Eq. (1) with 30,000 cells measured for each concentration.

L-MDR1/LLC-PK1 system (higher inhibition ratio and presumably lower P-gp expression) we selected the former for assessing P-gp inhibition by PIs and NNRTIs. MK571 and FTC had no influence on rhodamine123 accumulation in either cell line excluding possible influences of MRPs or BCRP on the assay. However, because MK571 quenched rhodamine123 fluorescence (see below) an increase in intracellular rhodamine123 could have been masked. We therefore also tested the non-selective MRP-inhibitor probenecid, which did not increase intracellular rhodamine123 fluorescence confirming the absence of an influence of MRPs on the assay.

Most PIs and NNRTIs inhibited P-gp in this flow cytometric assay. Lopinavir was the most potent inhibitor followed by saquinavir, atazanavir, and delavirdine (Table 3). The inhibitory effect of efavirenz was superimposed by cytotoxic effects at higher concentrations and was therefore not quantified. In the case of amprenavir and nelfinavir plateau effects were not reached and IC_{50} could not be calculated. In this assay ritonavir (tested only up to the solubility limit of 5 μM), nevirapine, and tipranavir had no effects nor had the NRTIs or tenofovir.

Most compounds tested did not quench rhodamine123 fluorescence. However, slight quenching effects (10–20% decrease in rhodamine123 fluorescence) were observed for atazanavir, lopinavir, nelfinavir, saquinavir, and FTC and distinct quenching effects (>20–40% decrease in rhodamine123 fluorescence) for tipranavir, abacavir, and MK571. For the latter the corresponding rhodamine123 efflux data were therefore not analysed.

4. Discussion

4.1. *P-gp inhibition by PIs*

It is widely accepted that PIs are substrates and most of them also inhibitors and inducers of P-gp [9–15]. However, the reported inhibitory potencies of individual compounds differed considerably between publications and the comparison of findings from independent studies was hampered by differences in the cell systems and methods used to quantify inhibition. Moreover, the very poor solubility of the PIs was

Table 3 – IC_{50}-values for PIs, NNRTIs, NRTIs, tenofovir, and tenofovir DF in the rhodamine123 efflux

Compound	IC_{50} in P388/dx (μM)
LY335979	0.033 ± 0.013
Quinidine	10.1 ± 2.4
Amprenavir	N.D.[a]
Atazanavir	24.9 ± 5.6
Indinavir	No inhibition
Lopinavir	5.5 ± 2.8
Nelfinavir	N.D.[a]
Ritonavir	No inhibition
Saquinavir	12.5 ± 3.0
Tipranavir	No inhibition
Delavirdine	38.1 ± 12.7
Efavirenz	N.D.[b]
Nevirapine	No inhibition
Abacavir	No inhibition
Didanosine	No inhibition
Emtricitabine	No inhibition
Lamivudine	No inhibition
Stavudine	No inhibition
Tenofovir	No inhibition
Tenofovir DF	No inhibition
Zalcitabine	No inhibition
Zidovudine	No inhibition

Data are expressed as mean ± S.D.; N.D., not definable.
[a] Calculation of IC_{50} was not possible due to lack of plateau effects.
[b] Inhibitory effect was superimposed by cytotoxic effects.

often neglected [10,32,33] and often test solutions beyond the solubility maximum were reported thus challenging the validity of the results [34]. So far, Perloff and his co-workers were the only group investigating most PIs in one assay without exceeding solubility maxima [13]. They tested PI effects on rhodamine123 efflux in LS180V cells at a concentration of 10 μM and found the following ranking order of inhibitory potency: amprenavir > lopinavir > atazanavir = nelfinavir = saquinavir > ritonavir (indinavir had no effect in this assay). These results do not match completely our rhodamine123 efflux data, where amprenavir was much less potent than lopinavir, atazanavir, and saquinavir. This can possibly be attributed to differences in the cell line and the absence of an appropriate control cell line to exclude possible influences of other transporters also expressed in this colonic cell line. Our data are supported by another study, where amprenavir was also less potent than saquinavir and nelfinavir [35].

Taken together our calcein assay data for the first time demonstrate that tipranavir is also a P-gp inhibitor with potency comparable to lopinavir, nelfinavir, and ritonavir.

So far, the clinical consequences of P-gp inhibition by PIs in vitro are largely unknown. Because most P-gp substrates are also substrates of CYPs, an enzyme family often inhibited by PIs [1,2], both systems may be affected and inferences from plasma pharmacokinetics may be inconclusive. However, two in vivo studies with digoxin, a compound not metabolised by CYPs, demonstrate substantially altered pharmacokinetics after administration of ritonavir [17,36] with profound modulation of digoxin distribution and renal clearance well compatible with an alteration of P-gp's efflux activity [17]. Similarly, the increase in azithromycin exposure observed with co-administration of nelfinavir [16] cannot be attributed to a CYP interaction and may rather be caused by modulation of active transport. Ritonavir has also been shown to promote indinavir distribution across barriers (into cerebrospinal fluid and semen) to an extent that cannot be fully explained by the concurrent metabolic interaction of the two PIs [37]. Another clinical trial demonstrated an increased systemic exposure to the essentially non-metabolised P-gp substrate fexofenadine after single-dose co-administration of ritonavir and lopinavir/ritonavir by 2.2- and 4.0-fold, respectively [38], which points to an interaction at the level of drug transport.

Likewise, the favourable pharmacokinetics of other PI combinations (e.g. ritonavir-boosted lopinavir) may not only be a result of interaction through CYPs, but also through P-gp, because P-gp limits the oral bioavailability as well as the penetration of PIs into sanctuary sites and into lymphocytes [3,39–41].

4.2. *P-gp inhibition by NNRTIs*

Data concerning the interaction of NNRTIs with P-gp are sparse. NNRTIs are not P-gp substrates [18,42–44] and efavirenz and nevirapine [18,44,45] are neither P-gp inhibitors. In contrast, delavirdine significantly inhibited rhodamine123 transport with an IC_{50}-value of 30 μM [18]. This matches our calcein and the rhodamine123 assay results, where delavirdine was the most potent (calcein assay) or only inhibitory NNRTI (rhodamine123 assay), and suggests that in vivo P-gp inhibition might only be important for drug interactions with delavirdine, although this has not been addressed in clinical trials yet. Results of such studies will be difficult to interpret because also delavirdine inhibits several CYPs [46] and also because P-gp induction by delavirdine [18] might superimpose inhibitory effects in vivo.

4.3. *P-gp inhibition by NRTIs, tenofovir, and tenofovir DF*

Only abacavir and tenofovir DF weakly inhibited P-gp whereas all other NRTIs and tenofovir had no interaction with P-gp. This is consistent with earlier results that found no P-gp inhibition by tenofovir in MDCKII-MDR1 cells [23] and by zidovudine, lamivudine, didanosine, and zalcitabine in colchicine-resistant cells [21] or only weak inhibition at high concentrations of zidovudine, didanosine, lamivudine, zalcitabine, and sanilvudine on calcein-acetoxymethylester efflux from L-MDR1 cells [32]. The weak P-gp inhibition by the tenofovir ester prodrug tenofovir DF and the lack of inhibition by tenofovir itself is also in line with earlier results demonstrating that in contrast to tenofovir, tenofovir DF is a substrate of P-gp [23–25].

4.4. *Comparison of the different methods applied for the quantification of P-gp inhibition*

Generally, the previously validated [27,31] calcein assay was the faster and more robust method of the two P-gp inhibition assays applied. On one microtiter plate 10 concentrations of a compound can be tested in octuplet each containing more than 10^6 cells. Consequently, interassay variability is small and concentration–response curves can be obtained at low cost over a large concentration range thus enabling calculation

of IC_{50} or *f*2-values for immediate comparison of inhibitory potencies. Moreover, the calcein fluorescence is bright, insensitive to pH, Ca^{2+}, and Mg^{2+} concentrations, and not susceptible to quenching effects [47,48].

Compared to the calcein assay the flow cytometry assay is more laborious, but allows at least intermediate throughput analysis. Because rhodamine123 is also transported with lower transport efficiency by MRPs [49], we tested the influence of MK571 and probenecid on this assay and found no influence in either cell system, confirming that these cells are lacking significant amounts of MRPs [50]. In agreement with the absence of rhodamine123 transport by the wild type form of BCRP [51,52] the BCRP inhibitor FTC had no influence on the rhodamine123 assay. Hence this assay is largely independent from the presence of other important drug transporters. The most significant limitation of the rhodamine123 efflux assay is its susceptibility to quenching by test compounds. If quenching effects occur, inhibition of P-gp will be underestimated and may even go undiscovered. Therefore, the strong P-gp inhibitory potency of tipranavir (as detected by the calcein assay) was possibly missed in the rhodamine123 efflux assay because tipranavir's substantial quenching effect likely superimposed the increase in intracellular rhodamine123 fluorescence. Also for abacavir, quenching effects are most likely the reason for the apparent discrepancies between the calcein and rhodamine123 assay. Moreover, in the rhodamine123 efflux assay higher concentrations are often necessary to inhibit P-gp thus concealing P-gp inhibition by weak inhibitors or compounds which can only be tested at low concentrations like ritonavir.

Taken together, for the assessment of P-gp inhibition the calcein assay has several advantages in terms of suitability and feasibility over the rhodamine123 assay.

4.5. *Evaluation of different cell systems as a model for P-gp*

Comparison of the three different cell systems used in the calcein assay revealed a remarkable concordance in the ranking order of inhibition (Fig. 2, depicted only for P388/dx and pBCECs) and demonstrated that absolute *f*2-values depend on the cell line used. The absolute values were smallest in pBCECs and highest in L-MDR1 cells. There are two possible explanations for this phenomenon: First, species differences between human, murine, and porcine P-gp might affect drug binding to the transporter and thus also the extent of interaction. If this holds true, murine P-gp would generally exhibit a lower affinity to inhibitors than porcine P-gp and human a lower affinity than murine P-gp. An even more likely cause are differences in the expression level of P-gp. pBCECs as primary, untreated cells certainly express the lowest amount of P-gp resulting in low *f*2-values, whereas in the over-expressing cell lines higher concentrations of the inhibitors are necessary to completely block the transporter. The high expression level of P-gp in L-MDR1 cells also restricts the practicability of this cell model for assessing P-gp inhibition, because many compounds and especially those with limited solubility do not reach *f*2-values in this system [31]. Moreover, in contrast to the P388/dx/P388 system, some compounds also increase calcein-fluorescence in the parental cell line LLC-PK1, as observed, e.g. with amprenavir, atazanavir, ritonavir, tipranavir, and delavirdine in this study or with norgestimate in a previous study [29] pointing to the likely contribution of another transporter or of unspecific effects in this cell system that may disturb reliable evaluation of inhibitory potencies. Also in the rhodamine123 efflux many compounds revealed an effect in LLC-PK1 cells indicating the influence of porcine pgp or of other transporters that may also transport rhodamine123 in these cells [53].

The influence of other transporters can also hamper the results obtained with the calcein assay in pBCECs that lack a control cell line without P-gp expression. Although a substantial modulation of the calcein assay by MRPs and BCRP have earlier been excluded [27,54], an effect seen in pBCECs cannot be attributed to P-gp with certainty neither qualitatively nor quantitatively. Generally, an ideal cell system would consist of a parental cell line, which expresses no protein transporting the fluorescent substrate, and a corresponding over-expressing cell line differing from the parental cell line only in the expression of the respective transporter.

For all these reasons, among the cell lines tested the P388/dx/P388 system might be the most suitable for assessing P-gp inhibition, although murine mdr1a/1b and not human MDR1 is expressed and although P388/dx cells were not generated by transfection but by induction with doxorubicin. However, so far, there is no evidence, that P388/dx cells differ from P388 cells in the expression of transporters other than mdr1a/1b.

However, these results do not exclude that other cell systems like the MDCK/MDCK-MDR1 [55] might be as suitable as or even better screening systems than the P388/dx/P388 system. Moreover and as always, proof of the significance of such in vitro findings for an in vivo situation can only be obtained in appropriate clinical studies.

4.6. *Conclusion*

There is increasing evidence from clinical studies that the modulation of P-gp activity by co-administered antiretroviral drugs may substantially contribute to pharmacokinetic interactions and thus modify both effectiveness and safety of combination therapy. Our in vitro study compared the P-gp inhibitory potency of the vast majority of the antiretrovirals currently used to treat HIV infection. It is the first study to provide comprehensive information on their interaction profile under identical assay conditions and reveals that many compounds may indeed inhibit P-gp substantially. These results therefore suggest that many antiretrovirals (and not only PIs) may inhibit this important target (e.g. delavirdine). Our study further provides a basis for a comparison of the validity of different widely used assays and cell systems and indicates that among the systems tested, the calcein assay using the P388/dx/P388 cells may be the most suitable and reliable in vitro model for the quantification of P-gp inhibition.

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