Inhibition of MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3 by nucleoside, nucleotide, and non-nucleoside reverse transcriptase inhibitors

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Abbreviations: abacavir, [4-(2-amino-6-cyclopropylamino-9H-purin-9-yl)-1-cyclopent-2-enyl]methanol; cLSM, confocal laser-scanning microscope/microscopy; CMF, 5-chloromethylfluorescein; CMFDA, 5-chloromethylfluorescein diacetate; delavirdine, N-[2-[4-[3-(1-m ethylethylamino)pyridin-2-yl]piperazin-1-yl]carbonyl-1H-indol-5-yl]methanesulfonamide; efavirenz, 8-chloro-5-(2-cyclopropylethynyl)-5-(trifluoromethyl)-4-oxa-2-azabicyclo[4.4.0]deca-7,9,11-trien-3-one; emtricitabine, 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-pyrimidin-2-one; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HPI, HIV-protease inhibitor; lamivudine, 4-
amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1H-pyrimidin-2-one; LY335979, zosuquidar; MF-SG, methylfluorescein-sulfoglutathione; MK571, (3-(3-(2-(7-chloro-2-quinoliny1)ethenyl)phenyl) ((3-dimethyl amino-3-oxo propyl)thio)methyl)thio)propanoic acid); MRP, human multidrug resistance-associated protein; nevirapine, 1-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b:2′,3′-e][1,4] diazepin-6-one; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; P-gp, P-glycoprotein; SEM, standard error of the mean; tenofovir, 1-(6-aminopurin-9-yl)propan-2-yloxyethylphosphonic acid.
Abstract

Many drug interactions with drugs used for the therapy of human immunodeficiency virus (HIV) occur at the level of different cytochrome P450 isozymes. Increasing evidence suggests that antiretrovirals may also modify activity and expression of active drug transport systems. Such interactions may alter drug absorption, elimination, and also drug distribution and reach clinical importance if thereby access to the target site is affected. Beyond P-glycoprotein the family of multidrug resistance-related proteins (MRPs/ABCCs) substantially contributes to the elimination of numerous drugs and their metabolites. Because the interaction of MRPs with non-HPI antiretrovirals has not been studied thoroughly, we investigated whether important non-nucleoside reverse transcriptase inhibitors (NNRTIs) (delavirdine, efavirenz, nevirapine), nucleoside reverse transcriptase inhibitors (NRTIs) (abacavir, emtricitabine, lamivudine), and tenofovir as a non-nucleotide reverse transcriptase inhibitor can interact with MRP1, MRP2, and MRP3 in vitro. Inhibition of these ABC-transporters was quantified by confocal laser-scanning microscopy using the 5-chloromethylfluorescein diacetate assay.

With the exception of abacavir, which had no effect on MRP3, all test compounds increased intracellular 5-chloromethylfluorescein-fluorescence in a concentration-dependent manner and this effect was observed in all over-expressing cell lines but not in the parental cell line indicating inhibition of MRP1, MRP2, and MRP3. In conclusion the present study provides the first evidence for a significant and concentration-dependent inhibition of MRPs by NNRTIs, NRTIs, and tenofovir, which was most pronounced for delavirdine, efavirenz, and emtricitabine suggesting that this might contribute to some of the known drug interactions impairing HIV therapy and also to the superior effectiveness of combination pharmacotherapy.
Infections with the human immunodeficiency virus (HIV) are typically treated with drug combinations consisting of at least three different antiretroviral drugs. Essential components of this highly active antiretroviral therapy (HAART) are the HIV protease inhibitors (HPIs), the non-nucleoside and nucleoside reverse transcriptase inhibitors (NNRTIs and NRTIs) as well as the nucleotide reverse transcriptase inhibitor (NtRTI) tenofovir. While this combination therapy substantially improves the clinical prognosis for patients infected with HIV it concurrently increases the risk for drug-drug interactions (de Maat et al., 2003; Piscitelli and Gallicano, 2001).

Many drug interactions with antiretrovirals, but by far not all and particularly not those with NRTIs, occur at the level of different cytochrome P450 isozymes (CYPs) (Dasgupta and Okhuysen, 2001; de Maat et al., 2003; Piscitelli and Gallicano, 2001). Indeed, increasing evidence suggests that antiretrovirals may also modify activity and expression of active drug transport systems. Such interactions may determine drug absorption, elimination, and also drug distribution and reach clinical importance if thereby access to the target site is affected.

For HPIs it is already well documented both in vitro and in vivo that they have multiple sites of interaction and inhibit CYPs (Eagling et al., 1997, Malaty and Kuper, 1999; Kumar et al., 1999; Granfors et al., 2006, Mikus et al., 2006) and ABC transporters like P-glycoprotein (P-gp; MDR1/ABCB1) (Bachmeier et al., Ding et al. 2004; Gutmann et al., 1999; Perloff et al., 2005; Profit et al., 1999; Sankatsing et al., 2004). Other important ABC transporters belong to the family of multidrug resistance-related proteins (MRPs/ABCCs) that substantially contribute to the elimination of numerous drugs and their metabolites (Borst et al., 2000). Because the interaction of MRPs with non-HPI antiretrovirals has not been studied thoroughly, we investigated whether important NNRTIs (delavirdine, efavirenz, nevirapine), NRTIs (abacavir, emtricitabine, lamivudine), and tenofovir as an NtRTI can interact with MRP1, MRP2, and MRP3. Inhibition of these ABC-transporters was quantified by confocal
laser-scanning microscopy using the 5-chloromethylfluorescein diacetate (CMFDA) assay

(Bogman et al., 2003).
Materials and Methods

Compounds

The anti-HIV drugs and LY335979 (zosuquidar) were kindly provided by the corresponding manufacturers. Stock solutions of test compounds were dissolved in DMSO, only tenofovir was dissolved in aqua bidest. The DMSO concentration in the assays never exceeded 1 % (v/v), a concentration which was found not to influence the results of the assay.

Cell Lines

As an in vitro model for human MRP1, MRP2, and MRP3 we used MDCKII/MRP1, MDCKII/MRP2, and MDCKII/MRP3 cells. All cell lines were generated by stable transfection of the corresponding cDNA into MDCKII cells (Evers et al., 1998a,b; Kool et al., 1999) and kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam). MDCKII/Par cells served as a control. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulphate (Invitrogen, Karlsruhe, Germany).

cLSM: CMFDA Accumulation Assay

The method for measuring the functional activity of MRPs was adopted from the CMFDA assay for MRP2 (Bogman et al., 2003; Lindenmaier et al., 2005) and extended to MRP1 and MRP3. The non-fluorescent lipophilic CMFDA (MobiTec, Göttingen, Germany) passively penetrates the plasma membrane. Inside the cells cytosolic esterases cleave off its acetate residues, thereby releasing the fluorescent and membrane-impermeable product 5-chloromethylfluorescein (CMF) which can react e.g. with glutathione to form fluorescent conjugates. This methylfluorescein-glutathione complex (MF-SG) is then actively secreted by MRP2 (Bogman et al., 2003) and also by MRP1 and MRP3 if present. Intracellular accumulation of the MF-SG in cells was analyzed with a DM IRE 2 TCS SP II cLSM from Leica (Bensheim, Germany) as published previously (Lindenmaier et al., 2005) with minor alterations. In brief, living cells (6 x 10^5) were seeded on coverslips in a closed
miniperfusion chamber (H. Saur, Reutlingen, Germany) directly before the experiment and
preincubated for 30 min with or without the test compound in darkness at 37 °C in 1 ml
transport buffer consisting of Hank’s balanced salt solution and 1 mM pyruvate (Invitrogen,
Karlsruhe, Germany) for energy supply. Pilot kinetic experiments had demonstrated that
maximum effects are reached within 30 min (data not shown). Subsequently, CMFDA in a
final concentration of 50 nM was added and incubated for 10 min. Data were analyzed
according to Lindenmaier et al. (2005). The experiments were performed at least in triplicate
on different days. All compounds were tested in the highest soluble concentration or up to
maximum concentration not provoking cytotoxic effects. The selective MRP inhibitor MK571
(Biomol, Plymouth Meeting, PA, USA) served as positive control at a concentration of 20 µM
as previously established (Bogman et al., 2003).

Quenching Test
Potential errors in the quantification of CMF due to quenching effects of test compounds was
excluded in a quenching assay by adding increasing concentrations of individual compounds
to aliquots of the cell lysate after incubation with 50 nM CMFDA. Comparison of the
fluorescence with control cell lysates without the respective compounds confirmed that none
of them showed any quenching effect on the fluorescence of CMF.

Cytotoxicity Assay
Apart from efavirenz, none of the compounds tested exerted cytotoxic effects as evaluated
with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). Due to
pronounced cytotoxic effects, efavirenz was tested in the cLSM assay only up to 10 µM.

Glutathione Assay
CMF requires the conjugation with glutathione to generate the fluorescent MRP substrate
MF-SG. To exclude that the compounds alter intracellular glutathione levels, glutathione
concentrations were measured in all cell lines before and after incubation with the
 corresponding compounds using the QuantiChrom Glutathione Assay Kit (BioAssay Systems,
Hayward, CA, USA). The assay was conducted according to the manufacturer’s instruction with $5 \times 10^4$ cells/100 µl. None of the compounds altered the intracellular glutathione level in the cell lines used.

**Statistical Analysis**

$P$ values were calculated by unpaired two-tailed $t$-test or by analysis of variance (ANOVA) with Dunnett’s multiple comparison test for post hoc pair wise comparison of the results with the corresponding control (without inhibitor). All statistical analyses were performed with GraphPad InStat, version 3.05, GraphPad Software (San Diego, CA, USA). A $p$ value of $\leq 0.05$ was considered significant.
**Results**

Comparison of the intracellular CMF-fluorescence in the four cell lines revealed significantly higher intracellular CMF-fluorescence in the parental cell line MDCKII/Par compared to the over-expressing cell lines ($p < 0.0001$), which did not further increase in the presence of the selective MRP inhibitor MK571 (Fig. 1). In contrast, in all over-expressing cell lines, the addition of MK571 led to a significant increase in intracellular fluorescence ($p < 0.0001$) demonstrating the suitability of this cell system for evaluating inhibition of MRP1, MRP2, and MRP3. The increase in fluorescence provoked by MK571 was generally highest in MDCKII/MRP1 and lowest in MDCKII/MRP3 cells indicating different expression levels of the MRPs or different affinities of substrate (MF-SG) or inhibitor (MK571) to the respective MRPs. With the exception of abacavir, which had no effect on MRP3, all test compounds increased intracellular CMF-fluorescence in a concentration-dependent manner and this effect was observed in all over-expressing cell lines but not in the parental cell line indicating inhibition of MRP1, MRP2, and MRP3 (Figs. 2, 3).

A comparison of the inhibitory potencies is difficult, because differences in solubility precluded testing of all compounds in the same concentration range. However, comparison of the effects observed at 10 µM (16.7 µM for delavirdine) (Fig. 4) revealed that delavirdine, emtricitabine, and efavirenz were the most potent inhibitors in all three cell lines. The P-gp selective inhibitor LY335979 had no statistically significant influence on the CMF-fluorescence in any of the cell lines tested indicating that P-gp did not influence the assay.
Discussion

Increasing evidence suggests that the challenging drug interactions in patients with HAART are often caused by more than one mechanism (de Maat et al., 2003) stressing the importance of knowing all potential targets involved and considering their complex interplay for dose individualization. We therefore aimed to systematically quantify the modulatory effect of frequently used non-HPI antiretrovirals on the important efflux transporters MRP1, MRP2, and MRP3 in vitro. The present study provides the first evidence for a significant and concentration-dependent inhibition of MRPs by NNRTIs, NRTIs, and tenofovir.

Thus far only very few clinical studies have addressed this issue. MRP2 inhibition by tenofovir might contribute to the known interaction between tenofovir and didanosine. Co-administration of these two antiretroviral drugs leads to an increase of the area under the didanosine concentration-time curve (AUC) by 44 % to 60 % (Kearney et al., 2005). This may occur through tenofovir-induced inhibition of the active uptake of didanosine into the proximal tubule cells the by human organic anion transporter 1 (Kearney et al., 2004; Zimmermann et al., 2006) or by inhibition of purine nucleoside phosphorylase, an enzyme involved in the degradation of didanosine (Ray et al., 2004). However, assuming that the MRP2 inhibitor didanosine is also a MRP2 substrate, the increase in didanosine AUC could also be achieved by inhibition of MRP2-mediated efflux in the tubular brush border membrane or in other tissues.

Inhibition of several MRPs could also have contributed to the life-threatening toxicity of the MRP substrate vinblastine in a patient with HIV-associated multicentric Castleman’s disease who was maintained on lamivudine, abacavir, and nevirapine (Kotb et al., 2006).

Beyond effects in tissues responsible for drug absorption, metabolism, or elimination, MRP inhibition by NNRTIs, NRTIs, and tenofovir in leukocytes might also lead to increased intracellular concentrations of other antiretroviral drugs being transported by MRPs. For some HPIs it has been demonstrated that they are transported by MRP1 and MRP2 and that their
intracellular concentrations depend on the activity of MRPs in the leukocytes (Huisman et al., 2002; Jones et al., 2001; Janneh et al., 2005). Inhibition of MRP-mediated efflux from the target cells could therefore contribute to the superior effectiveness of combination therapy compared to monotherapy.

Limitations: (1) Due to their tissue distribution and localization in polarized endothelia/epithelia the three MRPs investigated do not play identical roles in drug absorption, distribution, and excretion. MRP2 is localized in apical membranes and may thus lower the bioavailability in the gut and increase the excretion of its substrates into bile and urine. In contrast, MRP1 and MRP3 are expressed basolaterally and therefore exhibit opposite effects to MRP2 on their substrates (Schinkel and Jonker, 2003). Because of the differences in transport direction and location of the transporters inhibition of MPR2 in vivo is expected to have other effects than inhibition of MRP1 or MRP3 and it cannot be clarified in an in vitro setting which effect will prevail. (2) The concentrations effective in vitro are partly higher than therapeutic plasma concentrations. Moreover, efavirenz and delavirdine are highly plasma protein bound and extrapolation to in vivo situations should therefore consider free and not total plasma concentrations. However, compounds were tested in over-expressing cell lines where much higher concentrations are needed for inhibition than in cells or tissues with normal MRP activity because many inhibitors are also substrates of the respective transporter. Moreover, after oral administration concentrations achieved in the intestine are much higher than plasma concentrations possibly enabling MRP inhibition in the gut. (3) For all these reasons the ultimate proof of clinical relevance can only be obtained in a clinical study. In vitro data may, however, be helpful to discover an interaction, to understand a mechanism, to detect the need for such a trial, and to plan it appropriately by selecting the most relevant compounds. In many cases this is not an easy task because in vitro evidence was obtained in different studies using cell systems and markers that are hardly comparable. We therefore believe that the merit of this study is to provide comprehensive information on the interaction
of the commonly used antiretrovirals with a family of important drug transporters and that this information was gathered under identical assay conditions. This allows defining ranking orders of inhibition which is more meaningful and reproducible than absolute concentrations (e.g. IC$_{50}$ values), which strongly depend on assay conditions (Weiss and Haefeli, 2006). Therefore it can only be addressed in appropriate clinical studies, whether the inhibition of MRP1, MRP2, or MRP3 is clinically relevant.

In conclusion, it has become increasingly evident that the mutual interaction between antiretroviral agents is often a complex interplay of modified activities of several targets. The results of this in vitro study clearly demonstrate MRP1, MRP2, and MRP3 inhibition particularly by delavirdine, efavirenz, and emtricitabine suggesting that this might contribute to some of the known drug interactions impairing HIV therapy and also to the superior effectiveness of HAART.
Acknowledgments

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Figure legends:

Figure 1: Validation of the cLSM assay evaluating MRP1, MRP2, and MRP3 inhibition:
Comparison of the effect of the MRP-inhibitor MK571 on intracellular CMF-fluorescence in
the parental and three over-expressing cell lines. Data are expressed as mean ± SEM of n =
23-27 experiments. P-values were determined by unpaired two-tailed t-test with *** denoting
p < 0.0001 and n.s. p > 0.05.

Figure 2: cLSM assay assessing the concentration-dependent increase in intracellular CMF-
fluorescence in MCDKII/Par (control), MDCKII/MRP1, MDCKII/MRP2, and
MDCKII/MRP3 cells by NNRTIs. Positive control: 20 µM MK571; negative control: buffer
without inhibitor. Data are expressed as mean ± SEM with n = 3-5 experiments. P-values (*, p
< 0.05;**, p < 0.01) were determined by ANOVA with Dunnett’s multiple comparison test
for post hoc comparison of the results with the buffer control.

Figure 3: cLSM assay assessing the concentration-dependent increase in intracellular CMF-
fluorescence in MCDKII/Par (control), MDCKII/MRP1, MDCKII/MRP2, and
MDCKII/MRP3 cells by NRTIs and tenofovir. Positive control: 20 µM MK571; negative
control: buffer without inhibitor. Data are expressed as mean ± SEM with n = 3-5
experiments. P-values (*, p < 0.05;**, p < 0.01) were determined by ANOVA with Dunnett’s
multiple comparison test for post hoc comparison of the results with the buffer control.

Figure 4: Percentage inhibition of MRP1 (A), MRP2 (B), and MRP3 (C) by anti-HIV drugs
at a concentration of 10 µM (16.7 µM for delavirdine) compared to the positive control
MK571.
Figure 2A

Delavirdine

Mean CMF-fluorescence normalized to control

- MDCKII/Par
- MDCKII/MRP1
- MDCKII/MRP2
- MDCKII/MRP3

MK571 control
buffer control
0.17 μM
1.7 μM
3.3 μM
16.7 μM
33.4 μM

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Figure 2B

Efavirenz

Mean CMF-fluorescence normalized to control

- **MDCKII/Par**
- **MDCKII/MRP1**
- **MDCKII/MRP2**
- **MDCKII/MRP3**

Concentrations: 1 µM, 2.5 µM, 5 µM, 7.5 µM, 10 µM

Significance levels: **p < 0.01**
Mean CMF-fluorescence normalized to control

Figure 2C

- MK571
- Buffer control
- 10 μM
- 100 μM
- 200 μM
- 400 μM
- 500 μM
Figure 3A

Abacavir

A

Mean CMF-fluorescence normalized to control

buffer control 10 µM 100 µM 200 µM 400 µM 500 µM

MK571 buffer control 10 µM 100 µM 200 µM 400 µM 500 µM

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MK571 buffer control 10 µM 100 µM 200 µM 400 µM 500 µM

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MK571 buffer control 10 µM 100 µM 200 µM 400 µM 500 µM

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MK571 buffer control 10 µM 100 µM 200 µM 400 µM 500 µM
Figure 4A

Percentage inhibition of MRP1 compared to the positive control MK571

- MK571 [20 μM]
- Delavirdine [16.7 μM]
- Emtricitabine [10 μM]
- Efavirenz [10 μM]
- Lamivudine [10 μM]
- Tenofovir [10 μM]
- Abacavir [10 μM]
- Nevirapine [10 μM]
Figure 4C

Percentage inhibition of MRP3 compared to the positive control MK571

MK571 [20 µM]
Delavirdine [16.7 µM]
Efavirenz [10 µM]
Emtricitabine [10 µM]
Nevirapine [10 µM]
Abacavir [10 µM]
Lamivudine [10 µM]
Tenofovir [10 µM]