Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs

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Background: Various drug transporters of the ATP-binding cassette (ABC) family restrict the oral bioavailability and cellular, brain, testis, cerebrospinal fluid and fetal penetration of substrate drugs. MDRI P-glycoprotein (P-gp) has been demonstrated to transport most HIV protease inhibitors (HPI) and to reduce their oral bioavailability and lymphocyte, brain, testis and fetal penetration, possibly resulting in major limiting effects on the therapeutic efficacy of these drugs.

Objectives: To investigate whether the ABC transporters MRP1, MRP2, MRP3, MRP5 and breast cancer resistance protein 1 (Bcrp1) are efficient transporters of the HPI saquinavir, ritonavir and indinavir.

Methods: Polarized epithelial non-human (canine) cell lines transduced with human or murine complementary DNA (cDNA) for each of the transporters were used to study transepithelial transport of the HPI.

Results: MRP2 efficiently transported saquinavir, ritonavir and indinavir and this transport could be enhanced by probenecid. Sulfinpyrazone was also able to enhance MRP2-mediated saquinavir transport. In contrast, MRP1, MRP3, MRP5, or Bcrp1 did not efficiently transport the HPI tested.

Conclusions: Human MRP2 actively transports several HPI and could, based on its known and assumed tissue distribution, therefore reduce HPI oral bioavailability. It may also limit brain and fetal penetration of these drugs and increase their hepatobiliary, intestinal and renal clearance. MRP2 function and enhancement of its activity could adversely affect the therapeutic efficacy, including the pharmacological sanctuary penetration, of HPI. *In vivo* inhibition of MRP2 function might, therefore, improve HIV/AIDS therapy.

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Introduction

MDRI P-glycoprotein (P-gp) is a drug-extruding ATP-binding cassette (ABC) transporter that efficiently transports most HIV protease inhibitors (HPI) [1–4]. P-gp is present in the apical membrane of many critical epithelia and endothelia. Because of its localization and distribution, P-gp limits the oral bioavailability and brain, testis and fetal penetration of HPI and in all likelihood lymphocyte penetration as well [1,3–8]. P-gp activity thus contributes to lower plasma levels and pharmacological sanctuary sites for HPI and may in this manner limit their therapeutic efficacy against HIV/AIDS.

Given the impact of P-gp on *in vivo* HPI pharmacology, it is important to assess whether other drug transporters of the ABC family can also efficiently transport HPI. For instance, *MRP1* encodes a widely expressed transporter [9] that, when present in epithelial cells, is found primarily in the basolateral membrane. In mice, it greatly reduces the accumulation of the substrate drug etoposide in cerebrospinal fluid [10,11]. The related transporter MRP2 is found in the apical membrane of several epithelia [12–14]. In rats, MRP2 contributes to hepatobiliary, intestinal and renal drug excretion and to the reduction of oral availability of its substrates [15,16]. Based on parallels with P-gp, it might reduce fetal and brain penetration of its substrates.

The breast cancer resistance protein (BCRP) is also an apical transporter in epithelia [17,18]. Pharmacological inhibition experiments in mice indicate that the mouse BCRP homologue Bcrp1 reduces oral bioavailability, limits fetal penetration and mediates hepatobiliary excretion of its substrates [19]. Clearly, if HPI are efficiently transported substrates of MRP1, MRP2 and/or BCRP, this may well affect their pharmacological disposition and thus their therapeutic efficacy.

Some reports suggest that MRP1 may be a transporter of saquinavir, ritonavir and perhaps indinavir [20–22]. There is, however, no direct experimental evidence that MRP2 transports HPI, although there are some indications that HPI can inhibit MRP-like transport in fish renal tubules and rat brain microvessels [13,23].

In order to assess directly whether HPI are transported substrates for MRP1, MRP2 and Bcrp1, a well-defined system was used consisting of polarized non-human (canine) MDCKII cells, transduced with human *MRP1* or *MRP2* complementary DNA (cDNA) or with murine *Bcrp1* cDNA [19,24,25]. More recently discovered members of the MRP family include MRP3 and MRP5. Expression of MRP3 is found in liver, adrenal gland, pancreas, kidney and intestine, whereas MRP5 is expressed in most tissues. Transport was also studied in transduced cell lines overexpressing human MRP3 and MRP5 [26,27]. Both MRP3 and MRP5 localize basolaterally in epithelial cells, but their *in vivo* pharmacological function is as yet unclear [28].

Materials and methods

Chemicals

Ritonavir (Norvir, 80 mg/ml) was from Abbott Laboratories Inc. (Abbott Park, Illinois, USA). [^{3}H]-Ritonavir and [^{3}H]-indinavir were obtained from Moravek (Brea, California, USA). Indinavir was obtained from Merck (West Point, Pennsylvania, USA). [^{14}C]-Saquinavir and saquinavir were provided by Roche Discovery Welwyn (Welwyn Garden City, UK). PSC833 [29] was a gift of Novartis Pharma Inc. (Basel, Switzerland) and GF120918 [30] was provided by GlaxoSmithKline (Research Triangle Park, North Carolina, USA). MK571 was from Biomol (Plymouth, Pennsylvania, USA) and probenecid was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Cyclosporin A (Neoral, 100 mg/ml) was from Novartis Pharma B.V. (Arnhem, the Netherlands).

Cell lines and transport assays

Generation and culturing of the polarized non-human (canine) MDCKII [31] cell lines stably expressing human *MRP1*, *MRP2*, *MRP3*, *MRP5* and murine *Bcrp1* have been previously described [19,24–27,32]. Transport assays were performed as described previously [8]. The amount of HPI in the cell layer at the end of the experiment was determined by liquid scintillation counting of the excised filter, after washing with ice-cold phosphate-buffered saline.

Statistics

P values for increased net drug transport were determined by the Student's t-test (one-tailed, assuming equal variance). The net drug translocation (i.e., the difference between apically and basolaterally directed transport) after 4 h was used to compare transport rates.

Results

Transport of protease inhibitors by multidrug resistance proteins

MDCKII parental cells and derived cell lines stably transduced with different MRP cDNA were cultured on porous membranes in Transwell plates to test for their capacity to mediate transepithelial transport of 5 μmol/l [^{14}C]-saquinavir, [^{3}H]-ritonavir, or [^{3}H]-indinavir (Fig. 1). These cells form a polarized monolayer in which the rate of basolaterally or apically directed translocation can be followed after adding drug

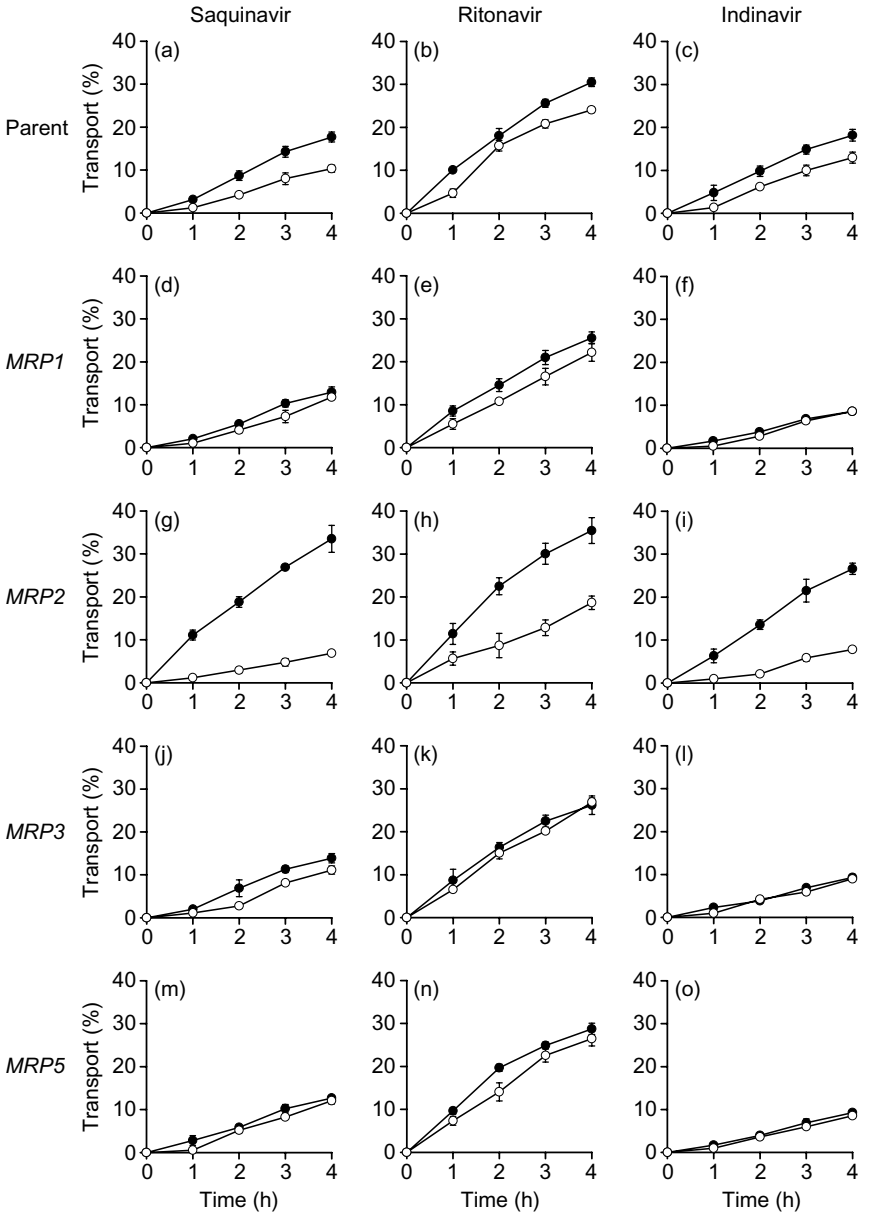

Fig. 1. Transepithelial transport of [^{14}C]-saquinavir (5.0 μmol/l), [^{3}H]-ritonavir (5.0 μmol/l) or [^{3}H]-indinavir (5.0 μmol/l) in MDCKII (parent), MDCKII–*MRP1*, MDCKII–*MRP2*, MDCKII–*MRP3* or MDCKII–*MRP5* monolayers in the presence of 1 μmol/l GF120918. At the start of the experiment, radioactive drug was applied to one compartment , and the percentage of radioactivity appearing in the opposite compartment was measured at 1, 2, 3 and 4 h. Results are expressed as mean values (n = 3), with bars indicating the SD (for some values the range is smaller than the size of the symbols used). Translocation from the basolateral to the apical compartment (●); translocation from the apical to the basolateral compartment (○).

to the apical or basolateral side of the monolayer. Without active transport, passive translocation of drug will be similar in both directions. Based on the subcellular localization of each transporter in polarized cells, MRP1, MRP3 and MRP5 should transport their substrates in the basolateral direction, and MRP2, Bcrp1 and P-gp in the apical direction. The P-gp inhibitor GF120918 (1 μmol/l) was present to suppress the moderate apically directed HPI transport mediated by endogenous P-gp in the parental cell line (Fig. 1 a–c). Even 5 μmol/l GF120918 did not significantly inhibit MRP1 or MRP2 [33]. Under these circumstances, the *MRP1*-, *MRP3*- and *MRP5*-transduced lines did not mediate substantial polarized transport of any HPI (Fig. 1d–f, j–l and m–o, respectively). However, MRP2 was clearly able to mediate substantial apically directed transport of all tested HPI (Fig. 1g–i; $P < 0.005$). Of the three HPI, saquinavir was transported most efficiently by MRP2, and ritonavir least. Drug concentrations were determined by high-pressure liquid chromatography coupled to an electrospray ionization sample inlet and two quadrupole mass

analyzers. This analysis indicated that $> 95\%$ of the translocated radioactivity represented unchanged drugs. Metabolites were not detected (data not shown). When HPI transport was studied in MDCKII–*Bcrp1* cells [19] under identical conditions to those described above except that 5 μmol/l PSC833 was used instead of 1 μmol/l GF120918 to suppress endogenous transporters, the results indicated that Bcrp1 is not an efficient transporter of the tested HPI (data not shown).

Enhanced multidrug resistance protein 2-mediated transport

Since MRP2-mediated transport of HPI might well have therapeutic relevance, a number of candidate MRP2 inhibitors were tested for their capacity to inhibit effectively MRP2-mediated transport of 5 μmol/l saquinavir, but without causing cytotoxicity. The following compounds were tested up to the concentrations indicated: MK571 (50 μmol/l), L-BSO (100 μmol/l for 24 h), PSC833 (10 μmol/l), ritonavir (50 μmol/l) and cyclosporine A (250 μmol/l), but none of these was able to meet these demands.

Unexpectedly, 500 μmol/l probenecid, one of the tested candidate MRP2 inhibitors, enhanced MRP2-mediated transport of the tested HPI dramatically. There was also an increase in saquinavir transport in the parental line, most likely caused by enhanced activity of the low level of endogenous canine MRP2 [31]. In an effort to saturate endogenous transporters, HPI concentration was increased from 5 to 50 μmol/l (Fig. 2a–c). Exposure of the cells to 500 μmol/l probenecid increased the net translocation in the parental cell line (Fig. 2g–i; $P < 0.001$), but this effect was much more pronounced in the *MRP2* line (Fig. 2j–l; $P < 0.05$).

To exclude the possibility that probenecid caused these effects by inhibiting an endogenous basolaterally located efflux system of HPI (e.g., MRP1-like), the percentage of total drug in the cell layer was determined after 4 h (Fig. 2; numbers in the panels). The intracellular saquinavir concentration was not altered significantly by probenecid in the parental line (Fig. 2a,g), whereas it decreased considerably in the *MRP2* line in the presence of probenecid (Fig. 2d,j; $P < 0.001$). This is consistent with a probenecid-enhanced efflux of saquinavir. Intracellular concentrations of ritonavir and especially indinavir were relatively low in all cell lines and, therefore, more difficult to interpret, but they clearly did not increase upon probenecid incubation.

To confirm that the observed enhanced transport was mediated by enhanced MRP2 function and to determine whether higher concentrations of probenecid can inhibit MRP2-mediated saquinavir transport, transport of saquinavir was studied in the presence of a wide concentration range of probenecid (Fig. 2m). A *Neomycin*-transduced subclone of MDCKII cells (*Neo*-line), which is known to contain hardly any endogenous canine MRP2 (not shown), was also included.

The relative rates of saquinavir transport were determined (i.e., the percentage of apically directed transport divided by basolaterally directed translocation after 4 h; Fig. 2m). Without probenecid, this relative rate was 6.3 in the *MRP2* line, compared with 2.0 and 1.3 in the parental and the *Neo*-line, respectively. The maximum relative rates of saquinavir transport with probenecid in the parental, *Neo*-line and *MRP2* line were, respectively, 5.6, 1.2 and 32. These data are in agreement with the relative levels of *MRP2* expression.

Another drug that enhanced MRP2-mediated saquinavir transport is the clinically used uricosuric agent sulfinpyrazone (Fig. 2n), which was tested over a concentration range from 0 to its maximum dissolvable concentration of 5 mM. It is apparent that sulfinpyrazone enhanced MRP2-mediated saquinavir transport at concentrations between 1 and 5 mmol/l, but in a less dramatic way than probenecid. Sulfinpyrazone also enhanced saquinavir transport in the parental line, again most likely through enhancement of endogenous MRP2 activity.

Discussion

Our data show that human MRP2 is a relatively efficient transporter of saquinavir, ritonavir and indinavir and that this transport can be markedly enhanced by the drugs probenecid and sulfinpyrazone. Human MRP1, MRP3 and MRP5 and murine Bcrp1 are not efficient transporters of the tested HPIs.

The fact that most tested HPI are transported by MRP2 can have important implications for their pharmacological use. In rats, Mrp2 has been demonstrated to contribute to hepatobiliary, renal and direct intestinal excretion of its substrates and to limit their oral bioavailability [12,15,16]. Human MRP2 activity is, therefore, likely to reduce the plasma levels of HPI by these same processes. One might consider applying MRP2 inhibitors, in order to improve plasma levels of these drugs. However, suitable specific and efficient MRP2 inhibitors are not yet available. The risk of unpredicted inhibition of MRP2 in patients as a result of coadministered drugs is presumably limited, as we found that a number of candidate inhibitors of MRP2 were not able to inhibit MRP2-mediated saquinavir transport.

Unexpectedly, our *in vitro* data show that MRP2-

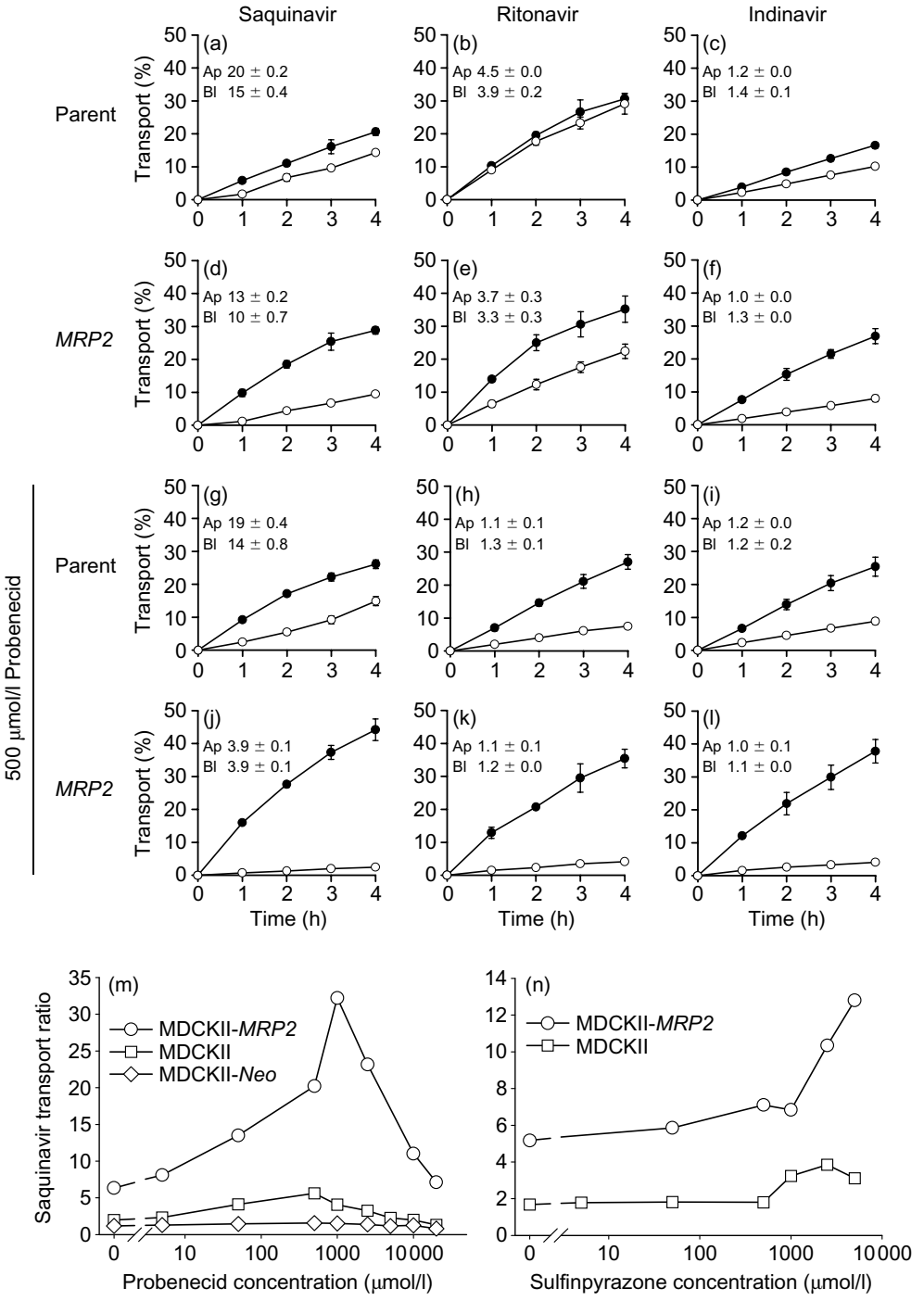

Fig. 2. Transepithelial transport of protease inhibitors. (a–l) Transepithelial transport of 50 μmol/l [^{14}C]-saquinavir, [^{3}H]-ritonavir, or [^{3}H]-indinavir in MDCKII (parent) or MDCKII–*MRP2* monolayers in the presence of 1 μmol/l GF120918, either without (a–f) or with (g–l) 500 μmol/l probenecid. At the start of the experiment, the radioactive drug was applied to one compartment (basolateral or apical), and the percentage of radioactivity appearing in the opposite compartment was measured at 1, 2, 3 and 4 h. Results are expressed as mean values (n = 3), with bars indicating the SD. Translocation from the basolateral to the apical compartment (●); translocation from the apical to the basolateral compartment (○). Numbers in the panels indicate the percentage of radioactivity retrieved from the cell layer 4 h after application of the drug to the apical (Ap) or basolateral (Bl) compartment. (m,n) Relative transport rates of [^{14}C]-saquinavir (5.0 μmol/l) measured after 4 h in the presence of 1 μmol/l GF120818 with increasing concentrations of (m) probenecid in MDCKII (low expression endogenous *MRP2*), MDCKII–*MRP2* (overexpression of human *MRP2*) and MDCKII–*Neo* (very low expression of endogenous *MRP2*) and (n) sulfinpyrazone in MDCKII and MDCKII–*MRP2* cells (n = 1). Relative transport rates were determined by dividing the percentage of apically directed transport by the percentage of basolaterally directed translocation.

mediated HPI transport can be boosted substantially by probenecid and somewhat by sulfinpyrazone. It may be that drugs other than probenecid can also enhance MRP2-mediated transport of HPI and could negatively influence oral bioavailability, plasma pharmacokinetics and sanctuary penetration of HPI.

One can envisage a relatively simple mechanism by which probenecid and sulfinpyrazone enhance MRP2-mediated HPI transport. Evers *et al.* [32] demonstrated that MRP2-mediated glutathione export can be enhanced by sulfinpyrazone and that sulfinpyrazone and glutathione are both transported by MRP2. They proposed that MRP2 has two substrate binding and transport sites, one binding the anionic glutathione (G-site) and the other binding drugs like sulfinpyrazone (D-site). Simultaneous occupation of both sites could then result in efficient export of both compounds. Probenecid and sulfinpyrazone are organic anions and it may be that they bind to the G-site and HPI to the D-site, resulting in efficient cotransport.

Our finding that MRP1 is not an efficient transporter for saquinavir, ritonavir and indinavir, appears to contrast with some previous reports [20–22]. Srinivas *et al.* [20] found that saquinavir and ritonavir can partially enhance doxorubicin sensitivity in an *MRP1*-overexpressing line, but the efficacy of these HPI in suppressing HIV replication in these cells was not substantially altered. The data, therefore, suggest that saquinavir and ritonavir can perhaps inhibit MRP1 but are not efficiently transported by it.

The studies of Jones *et al.* [21,22] were performed in CEM_{E1000} cells overexpressing *MRP1* as a result of extended selection with the cytotoxic drug etoposide, a procedure known frequently to result in a multitude of alterations in the cell, including upregulation of multiple different drug exporters [23]. It is, therefore, difficult to exclude that upregulation of other MRP-like HPI transporters (possibly MRP2) may have affected the data obtained.

In contrast, a study by van der Sandt *et al.* [34] presented data from polarized cells transfected with *MRP1* cDNA that was consistent with MRP1 not being a transporter of ritonavir and indinavir. They found that transport of ritonavir and indinavir towards the basolateral compartment was not increased relative to the untransfected parental cells [35]. Based on the available evidence and our own data, we do not want to exclude that MRP1 (and perhaps MRP3, MRP5 and Bcrp1 as well) are moderately active transporters of HPI, but it is clear that MRP2 is much more efficient. We, therefore, expect that the impact of MRP2 on HPI pharmacology will be more pronounced than that of the other transporters.

It might be worthwhile to determine if some herbal products or conventional drugs enhance MRP2 function in the way probenecid does, since this might lead to seriously decreased HPI exposure. If it turns out that *in vivo* MRP2 function has a prominent effect on HPI oral bioavailability and sanctuary penetration, as appears to be the case for P-gp, it may be useful to take this into account in further HIV/AIDS drug development programmes.

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