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P-Glycoprotein Limits Oral Availability, Brain, and Fetal Penetration of Saquinavir Even with High Doses of Ritonavir

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ABSTRACT

The low oral bioavailability of the HIV protease inhibitor (HPI) saquinavir is dramatically increased by coadministration of the HPI ritonavir. Because saquinavir and ritonavir are substrates and inhibitors of both the drug transporter P-glycoprotein (P-gp) and of the metabolizing enzyme CYP3A4, we wanted to sort out whether the ritonavir effect is primarily mediated by inhibition of CYP3A4 or P-gp or both. P-gp is known to limit the bioavailability, brain, testis, and fetal penetration of its substrates, so effective inhibition of P-gp by ritonavir in vivo might open up pharmacological sanctuary sites for saquinavir, with the potential of beneficial effects on therapy, but also of increased toxicity. In vitro, P-gp-mediated transport of saquinavir and ritonavir was only moderately inhibited by both HPIs compared with the potent P-gp inhibitor PSC833. When [^{14}C]saquinavir was orally coadministered with a maximum tolerated dose of ritonavir to wild-type and P-gp-deficient mice, saquinavir bioavailability was dramatically increased in both strains, but P-gp still limited the oral bioavailability of saquinavir, and its penetration into brain and fetus. These data indicate that in vivo, ritonavir is a relatively poor P-gp inhibitor. The highly increased bioavailability of saquinavir because of ritonavir coadministration most likely results from reduced saquinavir metabolism. Importantly, our data indicate that it is unlikely that ritonavir coadministration will substantially affect the contribution of P-gp to pharmacological sanctuary sites such as brain, testis, and fetus. Thus, if one wanted to effectively open these sites for therapeutic purposes, more efficient P-gp inhibitors should be applied.

HIV protease inhibitors (HPIs) play an important role in the currently used highly active antiretroviral therapy (HAART) in HIV-infected people. Although the introduction of HPIs has dramatically improved the disease prognosis, eradication of HIV has never been achieved (Finzi et al., 1997; Chun et al., 1999; Saag and Kilby, 1999). This may be related in part to several pharmacological limitations of HPIs. For instance, several HPIs, such as saquinavir have a low and variable oral bioavailability (Perry and Noble, 1998), which means that patients have to take HPIs frequently and at high dosages. This can lead to poor therapy adherence and associated low and variable HPI plasma concentrations (Rana and Dudley, 1999), which greatly enhances the chance of development of HPI-resistant HIV mutants.

It is known that when saquinavir is coadministered with the HPI ritonavir, its oral bioavailability increases dramatically in both animals and humans (Kempf et al., 1997; Kaufmann et al., 1998; Koudriakova et al., 1998; Cameron et al., 1999). Two mechanisms have been proposed to explain this finding. It has been demonstrated that inhibition of the metabolizing CYP3A4 and other cytochrome P450 isoforms by ritonavir is a major factor in the dramatically increased saquinavir bioavailability (Eagling et al., 1997; Fitzsimmons and Collins, 1997; Koudriakova et al., 1998; Cameron et al., 1999; Kumar et al., 1999) because saquinavir is known to be a very good CYP3A4 substrate and ritonavir a potent inhibitor of CYP3A4 function. A second proposed mechanism takes into account that the drug transporter P-glycoprotein (P-gp) may restrict the oral bioavailability of saquinavir. Most HPIs, including ritonavir and saquinavir, are P-gp substrates (Alsenz et al., 1998; Kim et al., 1998a,b; Lee et al., 1998; Polli et al., 1999) and ritonavir has even been reported to be a good or very good P-gp inhibitor (Alsenz et al., 1998; Lee et al., 1998; Gutmann et al., 1999; Profit et al., 1999). When saquinavir and ritonavir are coadministered, ritonavir could inhibit P-gp function, resulting in increased saquinavir bioavailability.

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ABBREVIATIONS: HPI, HIV protease inhibitor; HAART, highly active antiretroviral therapy; P-gp, P-glycoprotein; HPLC, high-performance liquid chromatography; AUC, area under the curve; Mdr1a/1b, multidrug transporter 1a and 1b.

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P-gp is an active drug-transporter of the ATP binding cassette transporter family with a very wide substrate range (Juliano and Ling, 1976; Higgins, 1992; Gottesman and Pastan, 1993). It is abundant in the apical membrane of many pharmacologically important epithelial barriers, such as the intestinal epithelium, the blood-brain and blood-nerve barrier, the blood-testis barrier, and the materno-fetal barrier formed by the placental trophoblasts. As P-gp transports its substrates in an outward (extracellular) direction, it will prevent HPIs from crossing the intestinal epithelium from the gut lumen, and from passing blood-brain, blood-nerve, and blood-testis barriers, and the materno-fetal barrier from the bloodstream (Choo et al., 2000; Kim et al., 1998a; Smit et al., 1999). Thus, P-gp function contributes to both low oral bioavailability of drugs, and to poor penetration into several pharmacological sanctuaries. Experiments with knockout mice deficient for the drug-transporting Mdr1a and Mdr1b P-gps (Mdr1a$^{-/-}$/1b$^{-/-}$ mice) have greatly contributed to these insights (Huisman et al., 2000). Although humans have only one drug-transporting P-gp, MDR1, this seems to fulfil the same functions as the mouse Mdr1a and Mdr1b P-gps (Thiebaut et al., 1987; Croop et al., 1989; Gottesman and Pastan, 1993).

If part of the pronounced effect of ritonavir on saquinavir pharmacokinetics is mediated by inhibition of P-gp, it could also mean that the effect of P-gp in preserving several pharmacological sanctuaries could be diminished by ritonavir coadministration. This could have both positive and negative consequences. It might increase the penetration of saquinavir (and possibly other HPIs) into brain, nerves, testis, and P-gp–containing lymphocytes, thus opening up potential sanctuary sites for HIV, but it might also lead to increased toxicity, for instance in the unborn child in pregnant women treated with HPIs. To assess the potential relevance of ritonavir as a P-gp inhibitor during HIV therapy, we have analyzed the pharmacological interactions between saquinavir, ritonavir, and P-gp both in vitro, making use of cell lines overexpressing P-gps, and in vivo, making use of the Mdr1a/1b knockout mouse model.

Materials and Methods

Chemicals. Both [^{14}C]saquinavir (41.3 μCi/mg) and saquinavir were provided by Roche Discovery Welwyn (Welwyn Garden City, UK). Ritonavir was purchased from Abbott Laboratories Inc. (Abbott Park, IL) as Norvir (80 mg/ml). Radiolabeled [^{3}H]ritonavir (1.5 Ci/mg) was obtained from Moravek (Brea, CA). Methoxyflurane (Metofane) was from Mallinckrodt Veterinary, Inc. (Mundelein, IL). Deionized water was obtained using the Milli-Q Plus system (Millipore Corp., Bedford, MA). Bovine serum albumin was from Roche Molecular Biochemicals (Mannheim, Germany). *Taq* DNA polymerase and deoxynucleoside triphosphates (dNTPs) were purchased from Life Technologies (Breda, The Netherlands).

Cell Lines and Tissue Culture. The pig-kidney cell line LLC-PK1 was obtained from the American Type Culture Collection (Manassas, VA) and cultured as described (Schinkel et al., 1995). The generation of the subclones of LLC-PK1 transfected with either human *MDR1*, mouse *Mdr1a*, or mouse *Mdr1b* was described previously (Schinkel et al., 1995). Cells were cultured in M199 medium supplied with L-glutamine (Life Technologies) and supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal calf serum (Life Technologies) (complete medium) at 37°C in the presence of 5% CO_2. The cells were trypsinized every 3 to 4 days for subculturing.

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Transport Assays. Cells were seeded on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) at a density of 2.0×10^6 cells per well in 2 ml of complete medium. Cells were grown for 3 days with one medium replacement after 1 day. Two hours before the start of the experiment, complete M199 medium was replaced from both compartments with Optimem medium (Life Technologies), without serum, either with or without 50 μM ritonavir, supplied from a Norvir stock solution. At t = 0 h the experiment was started by replacing the medium with fresh Optimem medium, either with or without 50 μM ritonavir and containing 5 μM [^{14}C]saquinavir (3 kBq per well) and [^{3}H]inulin (5 kBq per well) in the appropriate compartment. The latter compound was added to check for leakage through the cell layers. Cells were incubated at 37°C in 5% CO_2 and 50-μL aliquots were taken each hour, up to 4 h. The radioactivity was measured in these aliquots by the addition of 4 ml of scintillation fluid (Ultima-Gold; Packard, Meriden, CT) and subsequent liquid scintillation counting. Inulin leakage was tolerated up to one percent per hour per well. The percentage of radioactivity appearing in the opposite compartment, of the total amount initially applied, was measured and plotted. In case [^{3}H]ritonavir transport was studied, a concentration of 5 μM [^{3}H]ritonavir (4 kBq per well) was used and when appropriate 50 μM saquinavir as inhibitor. Finally, [^{3}H]inulin was replaced by [^{14}C]inulin (1 kBq per well). When PSC833 (Valspodar) was used as an inhibitor of P-gp function, the same protocol was followed, except that only 5 μM PSC833 was used.

Drug Distribution Studies. Mice used in all experiments were females between 10 and 14 weeks of age. Animals were housed and handled according to institutional guidelines complying with Dutch legislation under a 12/12 h light/dark cycle at a temperature of 22°C. Wild-type, Mdr1a$^{+/-}$/1b$^{+/-}$ and Mdr1a$^{-/-}$/1b$^{-/-}$ mice were of a 99% FVB genetic background. The mice received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. Ritonavir, [^{3}H]ritonavir or [^{14}C]saquinavir were administered, both intravenously and orally, at a volume of 2.5 μl per gram body weight, under light methoxyflurane anesthesia. For intravenous administration, [^{14}C]saquinavir was dissolved in an 8% ethanol, 4.2% glucose solution, and oral [14C]saquinavir was administered in a 16.4% ethanol/3% glucose/15.6% Cremophor EL solution. Both solutions contained a [^{14}C]saquinavir concentration of 2.0 μg/μl and by mixing [^{14}C]saquinavir and saquinavir in a 1:5 ratio, animals usually received 1 to 2 μCi (37–74 kBq) of the radiolabeled drugs at a saquinavir dosage of 5 mg/kg.

As a control vehicle for Norvir, a 43.0% (v/v) ethanol solution was used at a pH of 4.3. This vehicle resembles the matrix of liquid Norvir and contains Cremophor EL (105 mg/ml) (Sigma Chemical Co., St. Louis, MO), propylene glycol (0.25 mg/ml), peppermint oil (3.5 mg/ml), and water-free citric acid (2.8 mg/ml). Oral ritonavir was administered as a mixture of Norvir, ethanol, and water (2:3:3, v/v/v). Animals were sacrificed at the appropriate time points after the drug application by orbital bleeding under methoxyflurane anesthesia, followed by a cervical dislocation.

Tissues were collected and processed as previously described by Smit et al. (1999). Unchanged saquinavir was determined in plasma according to van Heeswijk et al. (1998), but unchanged fetal saquinavir concentrations were too low to be determined by HPLC and were for that reason determined as radioactive drug equivalent per weight. Genotype analysis was done by PCR, according to Smit et al. (1999).

Ritonavir Toxicity. The maximum tolerated dose ritonavir in both wild-type and Mdr1a/1b-deficient mice was determined by orally administering the appropriate volume of Norvir to the mice under light methoxyflurane anesthesia and subsequent continuous visual checking of the animals up to 4 h. Animals were rechecked after 6 h and terminated after 24 h.

Statistical Analysis. Student's two-sided, two-tailed t test was used to perform statistical analysis of differences between two sets of

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data. $P < 0.05$ was considered statistically significant. Unless indicated otherwise, errors are represented as S.E.M.

Results

Effect of Ritonavir on Polarized Saquinavir Transport and Vice Versa in LLC-PK1 Cells Containing Human MDR1, Mouse Mdr1a, or Mouse Mdr1b P-gp. To establish to what extent ritonavir inhibits P-gp-mediated transport of saquinavir and vice versa, we made use of the polarized pig kidney epithelial cell line LLC-PK1 and subclones stably transfected with human *MDR1*, mouse *Mdr1a*, or mouse *Mdr1b* cDNA. The transfected lines contain roughly comparable levels of MDR1, Mdr1a, or Mdr1b P-gp (Schinkel et al., 1995; Smit et al., 1998). Cell lines were grown to confluent monolayers on porous membrane filters, and polarized transepithelial transport of 5.0 μM [^{14}C]saquinavir was measured. Where indicated, 50 μM ritonavir, a concentration close to the solubility limit, was added to both compartments as an inhibitor. Serum-free medium was used to minimize confounding effects of saquinavir and ritonavir binding to serum proteins (Kim et al., 1998a).

Figure 1, left column, shows that the relative rate of saquinavir transport (i.e., the rate of apically directed transport divided by basolaterally divided translocation) in the parental LLC-PK1 line (probably mediated by endogenous porcine P-gp) was only 2.2, which is low compared with the *MDR1*-, *Mdr1a*-, and *Mdr1b*- expressing lines, which have minimum relative rates of transport of 21.

In the presence of 50 μM ritonavir, active saquinavir transport was fully inhibited in the parental line, resulting in equal translocation in both directions, and a decreased relative rate of transport to only 1.3 in the L-*Mdr1b* cells. In the L-*MDR1* and L-*Mdr1a* cells, ritonavir treatment diminished apically directed transport significantly, but the basolateral translocation of saquinavir was not significantly increased (Fig. 1, second column). The data indicate that saquinavir is efficiently transported by human *MDR1* and mouse *Mdr1a* P-gp, and that this transport is only moderately inhibited by ritonavir.

In an analogous series of experiments we studied polarized [^{3}H]ritonavir (5.0 μM) transport and the effect of 50 μM saquinavir as an inhibitor (Fig. 1, third and fourth columns). The relative rate of transport of ritonavir was only 1.2 in the parental LLC-PK1 line, but more than 20 in the L-*MDR1* and L-*Mdr1a* lines as evidenced by increased apical transport and greatly diminished basolateral translocation. The rela-

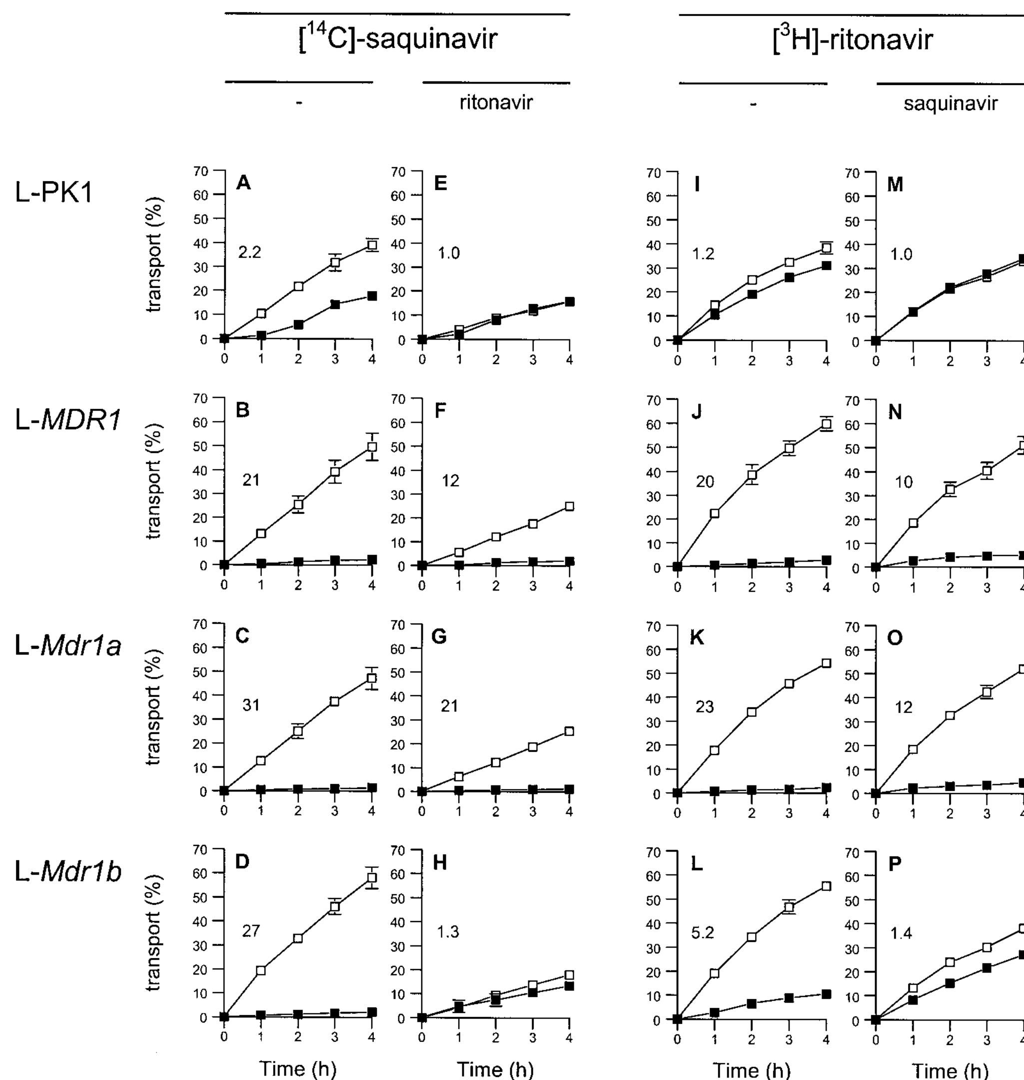

Fig. 1. Transepithelial transport of either [^{14}C]saquinavir (5.0 μM) or [^{3}H]ritonavir (5.0 μM) in LLC-*PK1*, L-*MDR1*, L-*Mdr1a*, or L-*Mdr1b* monolayers. When appropriate, unlabeled inhibitors, either saquinavir or ritonavir, were present in both compartments at a concentration of 50 μM. At t = 0, the radioactive drug was applied to one compartment (basolateral or apical) and the percentage of radioactivity appearing in the opposite compartment at t = 0, 1, 2, 3, and 4 h was measured and plotted. Data show a representative experiment (with $n = 3$) of three independent experiments. Results are expressed as mean values, with bars indicating the S.D. (for some values the range is smaller than the size of the symbols used). The figure next to the *y*-axis represents the relative transport ratio (e.g., the apical directed translocation divided by the basolateral directed translocation) at t = 4 h. □, translocation from the basolateral to the apical compartment; ■, translocation from the apical to the basolateral compartment.

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tive rate of transport was only 5.2 in the L-*Mdr1b* cells, because of high basolateral translocation, compared with the other two transfected lines. Addition of 50 μM saquinavir abrogated active ritonavir transport in the parental line and reduced the relative rate of transport to 1.4 in the L-*Mdr1b* line. In contrast, the relative rates of transport were reduced to only about 50% of their original values in the L-*MDR1* and L-*MDR1a* lines.

These data indicate that saquinavir and ritonavir are both efficiently transported by the MDR1 and Mdr1a P-gps, which are pharmacologically the most relevant isoforms. Ritonavir is somewhat more efficient in inhibiting apically directed saquinavir transport than vice versa, but for basolaterally directed transport, the situation may be reversed. Although inhibitor concentrations were close to the solubility limit and under serum-free conditions, inhibition was only partial. In contrast, when the transport of either 5.0 μM saquinavir or ritonavir was studied in the presence of 5 μM potent P-gp inhibitor PSC833, transport was virtually abrogated (data not shown). Together, the data show that both HPIs are only moderate MDR1 and Mdr1a P-gp inhibitors.

Saquinavir Clearance in Mdr1a$^{+/+}$/1b$^{+/+}$ and Mdr1a$^{-/-}$/1b$^{-/-}$ Mice. Despite saquinavir's being a good P-gp substrate, Kim et al. (1998a) previously found in a single-time point experiment that plasma radioactivity concentrations were not different between wild-type and Mdr1a$^{-/-}$ mice 4 h after intravenous injection of [^{14}C]saquinavir. To determine the effect of P-gp on the clearance of [^{14}C]saquinavir from plasma in more detail, we administered 5.0 mg/kg [^{14}C]saquinavir intravenously to *Mdr1a*$^{+/+}$/*1b*$^{+/+}$ (wild-type) and *Mdr1a*$^{-/-}$/*1b*$^{-/-}$ (knockout) mice and measured saquinavir plasma concentrations by HPLC and by total radioactivity at strategic time points over a 4-h period. Saquinavir clearance was rapid, and seemed to be P-gp independent at this dose, because there were no significant differences in the plasma concentrations between the two genotypes as determined by both HPLC and radioactivity measurements (Fig. 2a).

Saquinavir metabolism occurs very rapidly (Hsu et al., 1998), and it might also be (indirectly) affected by P-gp activity. However, the fraction of unchanged saquinavir over total radioactivity in plasma did not differ substantially between the two genotypes at any of the time-points (Fig. 2a). By comparing the AUCs of total radioactivity and unchanged saquinavir, it was calculated that, on the average, about 25 to 30% of the radioactivity in the plasma was present as unchanged saquinavir. However, this figure was only 10 to 20% at the 4-h time point. Strikingly, almost 45% of the radioactive dose was present in the liver at 2.5 and 5 min after administration, indicating rapid extraction of [^{14}C]saquinavir from plasma (Fig. 2b). The liver radioactivity concentration dropped considerably between 5 and 15 min, after which it decreased gradually. No significant differences were observed between wild-type and knockout mouse livers. In contrast to liver tissue, a considerable P-gp effect could be observed in brain tissue, where the [^{14}C]saquinavir brain penetration in wild-type, as determined by AUC till 4 h, was 181 ± 23 and 643 ± 126 h × ng/ml in knock-out brain tissue [i.e., a ratio of 3.6 between these two values (P = 0.002)].

Oral Uptake of saquinavir in Mdr1a$^{+/+}$/1b$^{+/+}$ and Mdr1a$^{-/-}$/1b$^{-/-}$ mice. In humans, low oral bioavailability of HPIs and especially saquinavir forms a problem in HIV therapy (Hsu et al., 1998). To assess the role that P-gp has in limiting the uptake of orally administered saquinavir, radiolabeled [^{14}C]saquinavir was given orally at 5 mg/kg to both wild-type and P-gp deficient mice, and plasma [^{14}C]saquinavir was measured between 5 min and 4 h after drug administration. The AUC_{PO} of [^{14}C]saquinavir in wild-types was 215 ± 22 and in knockout animals 349 ± 48 h × ng/ml. The oral availability (defined as AUC_{PO}/AUC_{IV} × 100%) of this dose of saquinavir was 9.1 ± 1.1% and 14.1 ± 2.2% in wild-type and P-gp deficient mice, respectively, as determined by total radioactivity (Fig. 3a, inset). Under these conditions, the concentrations of unchanged saquinavir in plasma were too low to be detected by HPLC. The data indicate that P-gp by itself has only a moderate, albeit significant (P = 0.02) effect on [^{14}C]saquinavir oral availability in mice.

Increased Saquinavir Oral Bioavailability by Ritonavir-Saquinavir Coadministration. To investigate to what extent inhibition of P-gp by ritonavir could contribute to the elevated saquinavir plasma concentrations seen in ritonavir-saquinavir coadministrations, we performed ritonavir-saquinavir oral coadministration experiments in wild-type and P-gp-deficient mice. We first determined the maximum tolerated oral dose of ritonavir in wild-type and P-gp-deficient mice, because we were aiming for maximal inhibitory effects of ritonavir. At 100 mg/kg, mice lacking functional P-gp showed severe signs of ataxia and tremor, suggesting a neurotoxic effect of ritonavir. Three out of four mice died within 2 h after drug administration at this dose. An oral dose of 50 mg/kg ritonavir was well toler-

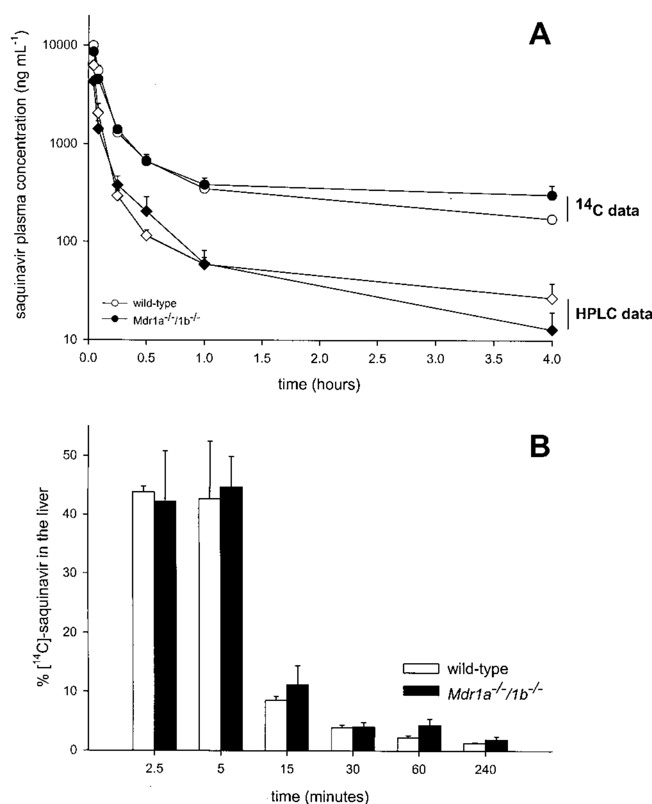

Fig. 2. Saquinavir plasma concentration and hepatic content after intravenous administration of 5 mg/kg [^{14}C]saquinavir to wild-type (open symbols) and Mdr1a$^{-/-}$/1b$^{-/-}$ animals (filled symbols). Error bars indicate the standard error of the mean (n = 4–5). A, saquinavir plasma concentration as determined by total radioactivity (circles) or HPLC detection (diamonds). B, percentage of the administered [^{14}C]saquinavir dose in time present in liver tissue as determined by total radioactivity.

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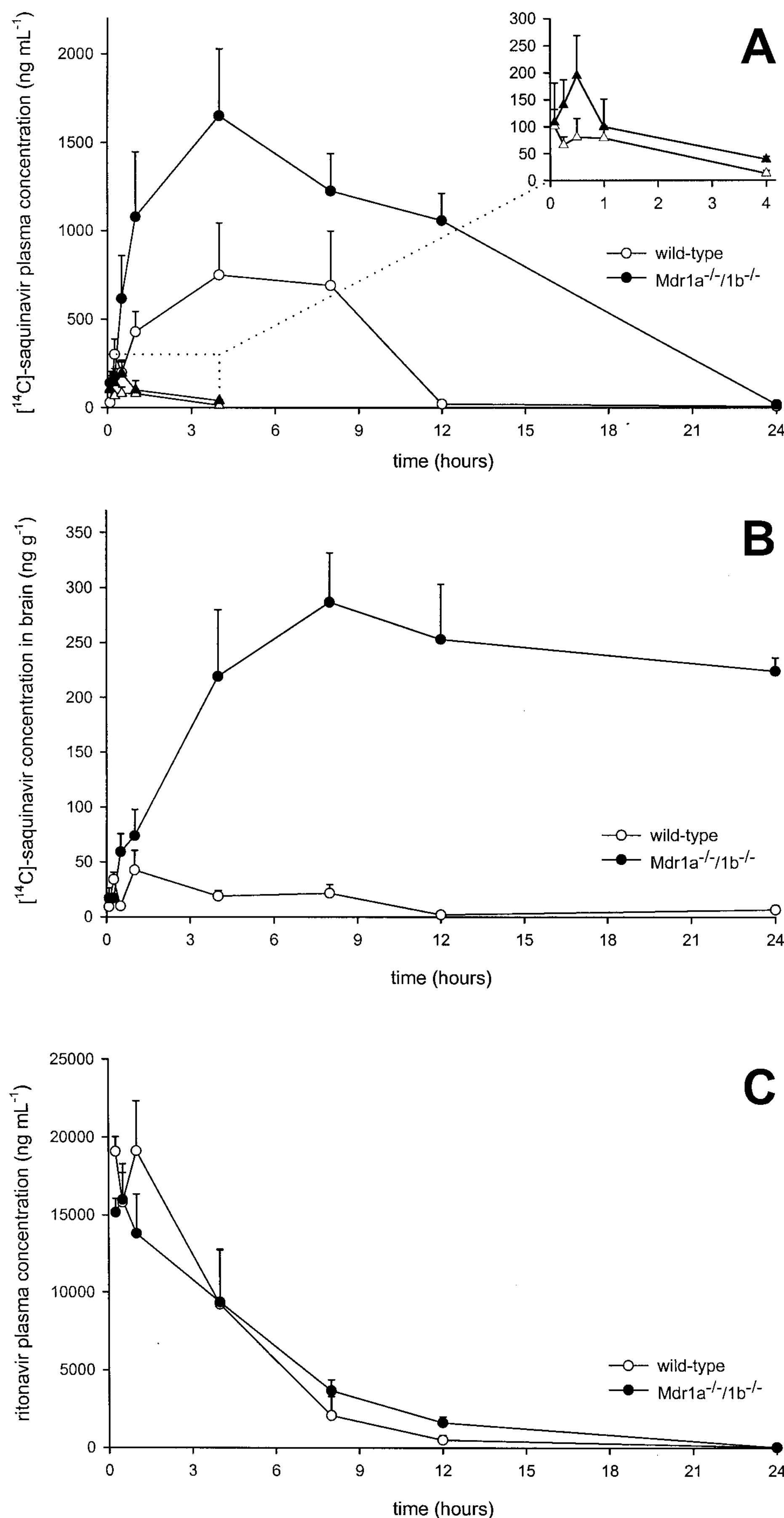

Fig. 3. Saquinavir plasma and brain concentrations in wild-type (open symbols) and Mdr1a$^{-/-}$/1b$^{-/-}$ animals (closed symbols) after oral administration of 5 mg/kg [^{14}C]saquinavir. Error bars indicate the standard error of the mean (n = 4 to 8). A, saquinavir plasma concentration in time as determined by total radioactivity. The upper two lines in the main panel indicate the saquinavir plasma concentrations of the animals which were coadministered with 50 mg/kg ritonavir 30 min before t = 0. The lower two lines, and the inset, indicate the saquinavir plasma concentrations of mice that received 5 mg/kg [^{14}C]saquinavir alone. B, saquinavir brain tissue concentration in time as determined by total radioactivity after oral coadministration of 50 mg/kg ritonavir 30 min before t = 0. C, ritonavir plasma concentration in time as determined by HPLC. Both genotypes received 50 mg/kg ritonavir orally 30 min before t = 0.

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ated by P-gp knockout mice up to at least 24 h. Wild-type mice showed only transient moderate signs of neurotoxicity at 400 mg/kg ritonavir, the highest dose tested. This demonstrates that P-gp-deficient mice are at least 4-fold more sensitive to oral ritonavir than wild-type mice. In a small-scale pilot experiment, similar plasma radioactivity concentrations were observed at 15, 30, and 60 min after administration of oral [^{3}H]ritonavir (50 mg/kg) to wild-type and knockout mice. However, [^{3}H]ritonavir seemed to penetrate somewhat more into brain tissue of P-gp-deficient mice (data not shown). Although we cannot definitely identify the cause of increased ritonavir sensitivity in P-gp knockout mice, increased CNS toxicity seems a likely possibility.

Based on the ritonavir toxicity data, for the ritonavir-saquinavir coadministration experiment, oral ritonavir was dosed at 50 mg/kg, followed 30 min later by an oral dose of 5.0 mg/kg [^{14}C]saquinavir. In this manner, P-gp in both gut epithelium, liver, and other excretory organs, and at blood-tissue barrier sites, such as blood-brain and maternal-fetal barriers, would be exposed to high ritonavir concentrations at the time of [^{14}C]saquinavir administration. The [^{14}C]saquinavir plasma concentration-time curves of wild-type and P-gp-deficient mice in this coadministration experiment are depicted in Fig. 3a. The data demonstrate a dramatic increase in [^{14}C]saquinavir oral availability due to ritonavir coadministration (from 9.1 ± 1.1% to 232 ± 70% in wild-type mice and from 14.1 ± 2.2% to 865 ± 105% in P-gp-deficient mice). The AUC of [^{14}C]saquinavir was 5 500 ± 1621 in wild-types and 21 423 ± 2 101 h/ng/ml in knockout animals ($P < 0.0001$). AUCs of unchanged saquinavir as measured by HPLC were 2198 ± 721 and 7876 ± 1424 h × ng/ml, respectively ($P < 0.0002$). Because large increases in bioavailability occurred in both wild-type and P-gp-deficient mice, inhibition of P-gp cannot have been a major factor in the increase. Despite the very high ritonavir dosage applied, P-gp-deficient mice still displayed a significantly higher availability of both total radioactivity and unchanged saquinavir ($P < 0.0002$) than wild-type mice. Note that the $AUCs_{0.5-24.5\ h}$ of ritonavir as determined by HPLC, were comparable between the wild-type and the P-gp-deficient animals [90,361 ± 14,600 and 98,960 ± 13,453 h × ng/ml, respectively (Fig. 3c)]. The data indicate that even at a very high oral ritonavir doses, inhibition of P-gp activity is not a major factor in the ritonavir-saquinavir pharmacokinetic interaction. The AUC fraction of unchanged saquinavir relative to total radioactivity was 40.0 ± 17.6% and 36.8 ± 7.6% in wild-type and knockout mice, respectively, showing that with oral ritonavir coadministration, a large fraction of the orally absorbed saquinavir is available as unchanged drug.

P-gp Still Limits Saquinavir Penetration into Brain after Oral Coadministration of Ritonavir and Saquinavir. P-gp in the blood-brain barrier has been demonstrated to limit the brain accumulation of [^{14}C]saquinavir (Kim et al., 1998a) and could thus contribute to the brain acting as a pharmacological sanctuary site for HIV. To see whether high-dose ritonavir could interfere with blood-brain barrier P-gp activity, we determined the brain concentrations of [^{14}C]saquinavir in the same animals in which the effect of ritonavir on saquinavir oral availability was studied (Fig. 3b). Comparison of Fig. 3, a and b, shows that the brain penetration and retention of [^{14}C]saquinavir in P-gp-deficient mice was far higher (18.7-fold; $P < 0.0001$) than could be explained by the somewhat higher plasma exposure in these mice (3.9-fold, $P = 0.0001$) over the 24-h period analyzed. Thus, high-dose ritonavir does not abrogate blood-brain barrier P-gp activity.

Placental P-gp Still Limits Saquinavir Penetration into Fetuses after Coadministration of Ritonavir and Saquinavir. Smit et al. (1999) have demonstrated that P-gp at the maternofetal barrier in the placenta is able to limit saquinavir penetration into mouse fetuses after an intravenous dose of 1 mg/kg [^{14}C]saquinavir to the mother and that this barrier function can be abrogated by treating the dams with the P-gp inhibitors GF120918 or PSC833. We wanted to establish whether in a clinically more realistic setting (i.e., after oral administration of saquinavir), placental P-gp also limits fetal saquinavir penetration. Moreover, we wanted to determine whether high-dose coadministered ritonavir could interfere with the placental P-gp barrier function, because coadministration of saquinavir and ritonavir may also be applied to pregnant women (Minkoff and Augenbraun, 1997). To address these questions, we generated fetuses of all three genotypes ($Mdr1a^{+/+}/1b^{+/+}$, $Mdr1a^{+/-}/1b^{+/-}$ and $Mdr1a^{-/-}/1b^{-/-}$) in a single mother by crossing heterozygous ($Mdr1a^{+/-}/1b^{+/-}$) dams to male $Mdr1a^{+/-}/1b^{+/-}$ mice (the *Mdr1a* and *Mdr1b* genes are directly linked and behave essentially as one genetic locus). Because the placental trophoblasts forming the maternofetal barrier are of fetal origin, the fetal genotype determines the P-gp expression at the placental barrier. At gestation day 15, pregnant dams received 50 mg/kg ritonavir orally (t = −30 min), followed by an oral dose of 5.0 mg/kg [^{14}C]saquinavir (t = 0 min). Four hours later, we determined the total fetal concentration and the maternal plasma concentration of [^{14}C]saquinavir. Fetuses were genotyped by PCR analysis. Figure 4 depicts the fetal tissue concentration of [^{14}C]saquinavir corrected for the maternal plasma concentration in fetuses of various genotypes. Penetration of [^{14}C]saquinavir into *Mdr1a/1b* null fetuses was nearly twenty times as high as penetration into wild-type fetuses ($P = 1.3 \times 10^{-10}$), whereas [^{14}C]saquinavir penetration into heterozygous fetuses did not differ significantly from penetration into wild-type fetuses. This indicates that despite coadministration of a high dose of ritonavir, and despite the high concentrations of plasma saquinavir over prolonged periods of time, saquinavir penetration into the fetuses was still very much limited by P-gp function. Thus, also placental P-gp activity was not abrogated by high ritonavir coadministration.

Discussion

This study demonstrates that saquinavir and ritonavir are both efficiently transported in vitro by human MDR1 and mouse Mdr1a P-gp, but that they are mutually only moderate inhibitors of the P-gp-mediated transport of each other. Consistent with this are our observations from in vivo mouse experiments, which also indicate that ritonavir at very high doses does not have a strong effect on the activity of P-gp toward saquinavir in the intestine, blood-brain barrier, and maternofetal barrier. Thus, as proposed before (Kempf et al., 1997; Kaufmann et al., 1998; Koudriakova et al., 1998; Steimer et al., 1998; Cameron et al., 1999), the dramatic effect of ritonavir on saquinavir oral availability and plasma concen-

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trations seems to be mainly caused by interference with saquinavir metabolism.

It has been suggested that ritonavir is a very good P-gp inhibitor (Gutmann et al., 1999), and several groups have reported that in vitro drug transport in isolated human lymphocytes and in the human Caco-2 cell line that was presumably due to P-gp activity, and in *MDR1*-overexpressing KB-V1 cells, could be extensively inhibited by ritonavir (Alsenz et al., 1998; Lee et al., 1998; Profit et al., 1999). In contrast, others have reported that ritonavir is a relatively poor P-gp inhibitor (Polli et al., 1999; Choo et al., 2000), based on interaction studies with [^{3}H]amprenavir and [^{3}H]indinavir. However, in our in vitro experiments, P-gp–mediated transport of saquinavir could be fully inhibited by the potent P-gp inhibitor PSC833, whereas ritonavir used at a 10-fold higher concentration was only able to partially inhibit P-gp function. The relatively modest inhibitory effect of ritonavir that we observed in L-*MDR1* and L-*Mdr1a* cells could relate to the high level of P-gp in these cells, which may make it more difficult to achieve complete inhibition. If P-gp levels in pharmacologically important barriers, such as the intestinal epithelium, the blood-brain barrier, and the maternofetal barrier are similarly high, this could explain the limited effect of ritonavir on P-gp activity that we observed in the in vivo mouse experiments. The dramatic increase in plasma saquinavir concentrations resulting from ritonavir-mediated inhibition of saquinavir metabolism complicates a direct comparison of in vivo P-gp activity toward saquinavir in the presence or absence of ritonavir. We therefore cannot exclude relatively small effects of ritonavir on P-gp activity in vivo.

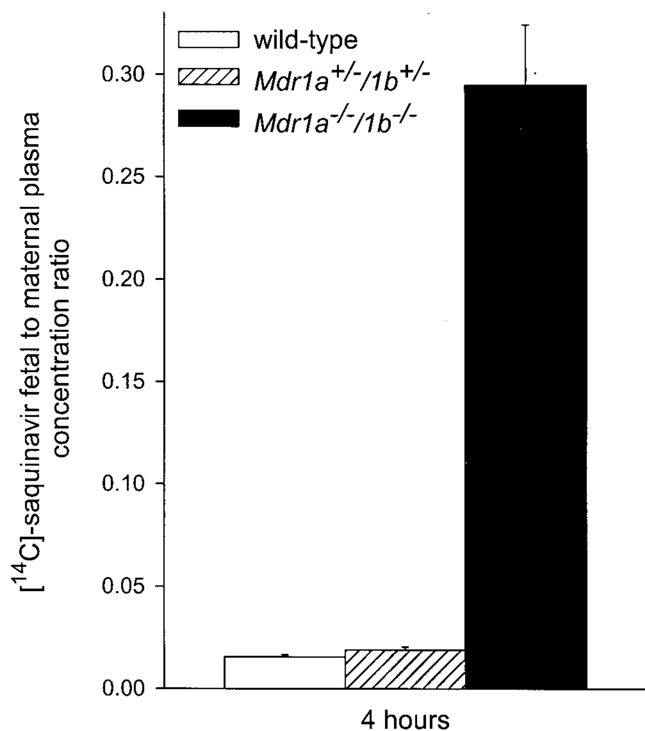

Fig. 4. Ratio of [^{14}C]saquinavir fetal concentration to maternal plasma concentration as determined by total radioactivity. Heterozygous mice were mated and received 50 mg/kg oral ritonavir and 5.0 mg/kg oral [^{14}C]saquinavir at gestation day 15. At 4 h after saquinavir dosing, dams were euthanized, and fetuses from five litters were collected. Maternal drug plasma concentration, fetal drug content, and genotypes were determined as described under *Materials and Methods*. Open bars indicate wild-type values, hatched bars heterozygous values, and filled bars Mdr1a$^{-/-}$/1b$^{-/-}$ values. Values are expressed as mean saquinavir concentration in fetal tissues (μg/g) normalized to maternal plasma saquinavir concentration (μg/ml). Ten to 26 fetuses of each genotype were analyzed. The average maternal saquinavir plasma concentration as determined by total radioactivity was 616 ± 96 ng/ml, 327 ± 53 ng/ml as determined by HPLC and the plasma ritonavir concentration was 3818 ± 559 ng/ml as determined by HPLC. Error bars indicate the S.E.M.

Previous experiments (Kim et al., 1998a; Smit et al., 1999) and this study indicate that P-gp activity has a strong restrictive effect on brain and fetal penetration of saquinavir, and a moderate effect on saquinavir oral bioavailability. This study demonstrates that all these effects are maintained upon coadministration of a very high dose of ritonavir, despite the high ritonavir plasma concentration and greatly increased plasma concentrations of unchanged saquinavir. It has been proposed that P-gp in the blood-brain barrier, the blood-testis barrier, and (a subset of) lymphocytes contributes to pharmacological sanctuary sites for HIV, resulting in enhanced likelihood of the development of HPI-resistant mutants and failure to eradicate infection (Kim et al., 1998a; Lee et al., 1998; Profit et al., 1999; Huisman et al., 2000). Moreover, poor oral bioavailability of HPIs because of P-gp could also contribute to low and variable plasma concentrations of HPIs and thus increase the chance of development of therapy-resistant viruses. In view of the reported P-gp-inhibitory activity of ritonavir, it was speculated that coadministration of ritonavir with saquinavir (and other HPIs) could open up these pharmacological sanctuaries, and thus improve HIV therapy. Our data suggest that, at least in brain and fetus, this is not the case; however, as explained above, it cannot be excluded that ritonavir inhibits P-gp activity in vivo slightly. We did not measure effects in lymphocytes in vivo, but because these cells probably contain lower levels of P-gp than blood-brain and placental barriers, there might be beneficial effects of ritonavir coadministration separate from the increase in plasma concentrations of saquinavir. Nevertheless, if brain/CNS and testis indeed do provide pharmacological sanctuaries for HIV, one should consider coadministration of dedicated, highly efficient P-gp inhibitors, such as PSC833 (Valspodar) or GF120918 (Boesch et al., 1991; Hyafil et al., 1993; Choo et al., 2000), to improve HPI penetration into these sanctuaries. These P-gp inhibitors have already been used in clinical trials of anticancer therapy to inhibit drug transporter proteins. Our data show that there would potentially be risks involved with such a strategy. The minimally 4-fold increased toxicity of ritonavir seen in P-gp knockout mice is a clear example. In our coadministration experiments, the ritonavir plasma concentrations were similar to those found in patients (Cameron et al., 1999). The dosage of ritonavir given to the mice was only 2-fold below the dosage at which the knockout mice suffered from severe toxicities; potentially, therefore, efficient blocking of P-gp function may also lead to ritonavir toxicity in humans.

It is likely that P-gp limits penetration of toxins into the developing fetus, both in mice and humans, because it is present in the placenta throughout development (MacFarland et al., 1994). Increased penetration of HPIs into the unborn child of HIV-positive pregnant women by using effective P-gp inhibitors would also be a point of concern, although there are two sides to this coin: on the one hand, during the critical early development stages, one would like to prevent exposure of the fetus to HPIs to prevent potential teratogenic effects (Olivero et al., 1997), because HIV transmission oc-

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curs only rarely during early pregnancy (Newell, 1998). On the other hand, most of the HIV infections of children of HIV-positive mothers occur during delivery (Newell, 1998), and it might be advantageous to "preload" the unborn child via the placenta shortly before birth with HPIs using P-gp inhibitors to have maximal prophylactic effect. Such preloading is currently already applied with zidovudine (AZT), which more readily penetrates the placenta (Casey and Bawdon, 1998).

It is clear that the concept of directed inhibition of P-gp activity to improve treatment of HIV with HPIs is still in its infancy, and careful clinical studies will be needed to establish whether such a strategy would indeed improve HPI efficacy, and whether it can be applied with sufficient safety. Our data do suggest that the current use of ritonavir in HPI coadministration regimens will have very limited impact on P-gp activity. Based on data previously presented by others and on data presented here, we consider it likely that almost all of the pharmacokinetic interaction effects of ritonavir should be attributed to its function as an inhibitor of CYP3A-mediated drug metabolism (Kumar et al., 1996; Kempf et al., 1997). Given the limited effect on P-gp, it is therefore probably safe to use the saquinavir-ritonavir drug combination in pregnant women, as long as the dramatic increase in plasma saquinavir concentration is taken into account.

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