0022-3565/02/3022-742–750$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 302, No. 2
4508/995632
Printed in U.S.A.

JPET 302:742–750, 2002

Effect of Interleukin-2 on Intestinal P-glycoprotein Expression and Functionality in Mice

CELINE VEAU, LAURENCE FAIVRE, SYLVIANE TARDIVEL, MIREILLE SOURSAC, HELENE BANIDE, BERNARD LACOUR, and ROBERT FARINOTTI

Unité Propre de Recherche de l'Enseignement Supérieur 2706 (C.V., L.F., S.T., M.S., H.B., B.L., R.F.); Laboratoire du Métabolisme Minéral des Mamifères, Ecole Pratique Hautes Etudes-Physiologie, Faculté de Pharmacie, Châtenay-Malabry (S.T., B.L.); and Service de Pharmacie Clinique et des Biomatériaux, Hôpital Bichat, Paris, France (R.F.)

Received September 4, 2001; accepted March 10, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

P-glycoprotein (Pgp), an active drug transporter expressed in enterocytes, can reduce intestinal absorption of drugs. Until now, interleukin-2 (IL2) has been reported as a Pgp modulator only in vitro. The present study examines the effects in vivo of IL2 after chronic treatment on intestinal Pgp protein expression and activity. This work also describes the effects of IL2 on the oral bioavailability of a Pgp substrate (digoxin) and of a Pgp/CYP3A cosubstrate (saquinavir). Human recombinant interleukin-2 (rIL2), administered to mice at 9 million international units/kg by intraperitoneal route twice daily for 4 days, led to a decrease in intestinal Pgp protein expression evaluated by Western blot with C219 antibody. In an in vitro everted gut sac model, rIL2 pretreatment decreased the Pgp-mediated transport of rhodamine 123 across mouse intestine by 37%. Moreover, rIL2 pretreatment markedly raised the area under the curve of orally administered digoxin from 3.5 ± 0.5 to 9.7 ± 1.5 mg min l^{-1} as a consequence of the reduction in intestinal Pgp activity. rIL2 treatment increased saquinavir bioavailability from 2.5 to 4.5%, showing that first-pass metabolism is not affected and that Pgp by itself has only a moderate effect on saquinavir oral bioavailability. In conclusion, rIL2 pretreatment reduces intestinal Pgp protein expression and activity in mice. However, the effect of such a treatment on drug bioavailability depends on the extent of their metabolism by CYP3A.

P-glycoprotein (Pgp) is an active drug transporter belonging to the ATP-binding cassette transporter family with a very wide substrate range. It is abundant in the apical membrane of many pharmacologically important epithelial barriers, such as the intestinal epithelium and the blood-brain barrier (Thiebaut et al., 1987; Lum and Gosland, 1995). In the intestine, Pgp transports its substrates in an outward (extracellular) direction, thus limiting their absorption from the intestinal lumen and contributing to the low bioavailability of such drugs (Terao et al., 1996; Wacher et al., 1996). In humans, Pgp is encoded by *MDR1*. In mice, *mdr1a* is the major RNA transcript expressed in small intestine (Schinkel et al., 1994).

The bioavailability of some drugs is also decreased by metabolism via a phase I metabolism involving cytochrome P450. CYP3A, extensively expressed in liver and in intestine of humans and rodents, is the main subfamily responsible for phase I metabolism of such drugs (Wacher et al., 1996). A sticking overlap of substrate specificity for CYP3A and Pgp has been observed (Wacher et al., 1995; Schuetz et al., 1996). Furthermore, Pgp and CYP3A share many inhibitors such as cyclosporin A, ketoconazole, and ritonavir. Hence, in most human and animal studies, simultaneous inhibition of Pgp and CYP3A by these compounds increases the bioavailability of Pgp/CYP3A cosubstrates (Kempf et al., 1997; Salphati and Benet, 1998).

Digoxin is a Pgp substrate poorly metabolized in humans and mice. Digoxin is considered as a reference substrate to test intestinal Pgp functionality in vivo (Greiner et al., 1999; Kawahara et al., 1999; Hoffmeyer et al., 2000). In humans, drug-drug interactions involving modulation of intestinal Pgp have been reported with digoxin: itraconazole or atorvastatin, two Pgp inhibitors, increase digoxin area under the curve (Sachs et al., 1993; Boyd et al., 2000). In mice, digoxin absorption is markedly increased in the absence of *mdr1a* (Mayer et al., 1996; Kawahara et al., 1999).

Saquinavir, a protease inhibitor used in combination in the treatment of HIV infection, has a very low bioavailability (4%) due to an extensive first-pass metabolism and an efflux by intestinal Pgp (Kim et al., 1998). In humans, saquinavir is

This work was supported in part by Chiron Laboratories (Suresnes, France).

ABBREVIATIONS: Pgp, P-glycoprotein; mdr, multidrug resistance; HIV, human immunodeficiency virus; IL2, interleukin-2; rIL2, human recombinant interleukin-2; MRP, multidrug resistance-associated protein; PBST, phosphate-buffered saline-Tween 20; PCR, polymerase chain reaction; AUC, area under curve; CL, clearance; F, oral drug bioavailability; Vd, volume of distribution; MRT, mean resident time; MIU, million international units; RT, reverse transcription; P450, cytochrom P450.

strongly metabolized by CYP3A4 (Noble and Faulds, 1996; Fitzsimmons and Collins, 1997).

Recently, interleukin-2 (IL2) has been used as immunomodulator in HIV treatment. This cytokine plays a central role in immune response by stimulation of Natural Killer cells and cytotoxic T-lymphocyte activity. Drug-drug interactions between human recombinant interleukin-2 (rIL2) and protease inhibitors have been reported previously (Piscitelli et al., 1998). A decrease in Pgp protein expression associated with a decrease in its mRNA by IL2 has been reported in human colon carcinoma cells (Stein et al., 1996). Therefore, IL2 could modify intestinal Pgp function in vivo and influence pharmacokinetics of Pgp substrates.

The aim of this study was to investigate the effects of IL2 pretreatment in mice on the activity and expression of intestinal Pgp, both at the mRNA and protein levels. These effects were correlated to the bioavailability of two Pgp substrates: digoxin (poorly metabolized) and saquinavir (strongly metabolized). Protein expression was estimated by Western blot using C219 as Pgp antibody. mRNA *mdr1a* expression was quantified by RT-PCR. To estimate Pgp activity, we used an in vitro model of everted gut sacs with rhodamine 123 as substrate for the transporter. Pharmacokinetics of digoxin was investigated after oral administration as reference. Bioavailability of saquinavir was investigated after oral and intravenous administration in mice.

Materials and Methods

Chemicals. rIL2 (18 MIU; Proleukin) was a gift from Chiron Laboratories (Suresnes, France). Saquinavir (200-mg capsules; Invirase) and saquinavir mesylate were from Roche Applied Science (Neuilly-sur-Seine, France). Digoxin Nativelle was from Procter & Gamble (Neuilly-sur-Seine, France). Cyclosporin (Sandimum) was from Novartis (Basel, Switzerland). (Carboxymethyl)cellulose, rhodamine 123, verapamil, probenecid, boric acid, and trifluoroacetic acid were from Sigma-Aldrich (St. Quentin Fallavier, France). Glucose (5%) was from MacoPharma Laboratories (Touroing, France). Methanol and acetonitrile were from Fisher Chemicals (Elancourt, France). Phosphoric acid (100%) and acetic acid (100%) were from Prolabo (Fontenay sous Bois, France).

Animals. Male adult mice (Swiss NMRI) weighing 28 to 30 g were obtained from Iffa Credo (L'Arbresle, France). Mice were fed standard laboratory ad libitum during 1 week before experiments.

Animal handling and experimentation were performed in accordance with the guidelines issued by the European Economic Community, as published in the Journal Officiel des Communautés Européennes (December 18, 1986; authorization L3600).

Animal Treatment. Mice received 9 MIU/kg of rIL2 by i.p. injection twice daily (at 9.30 AM and at 5.30 PM) for 4 days. Lyophilized rIL2 preparation was dissolved in 1.2 ml of sterile water and completed to 10 ml with 5% glucose to obtain a final concentration of 1.8 MIU/ml of rIL2. Control mice received vehicle alone (0.15 ml of 5% glucose i.p. twice daily for 4 days). Animals were fasted overnight, and all experiments were performed on day 5.

In Vitro Transport of Rhodamine 123 across Mouse Everted Gut Sacs. After decapitation of mice and laparotomy, the ileum was taken above the caecum, washed with isotonic saline, and everted. It was cut into 5-cm-long segments that were ligated at one end, filled on the serosal side by the free end with a rhodamine 123 solution (25 μM rhodamine 123 in buffer A), and tightly ligated to create a gut sac. Immediately, this everted sac was placed in 40 ml of buffer A (117.6 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11 mM glucose, and 4.7 mM KCl, pH 7.4). The solution was gassed by 95% O_2/5% CO_2 and maintained at 37°C throughout the experiment. Transport of rhodamine 123 from the serosal to the mucosal side was measured by sampling 500 μl of the external medium every 20 min up to 80 min. The rate of rhodamine 123 transport was expressed as percentage secreted per minute in the mucosal compartment.

In some experiments, inhibitors were added in the mucosal and the serosal sides at the same concentration. Several inhibitors of different intestinal drug transporters were used: verapamil (25 and 250 μM) and cyclosporin A (10 and 50 μM) as Pgp blockers and probenecid (25 and 100 μM) as a multidrug-resistance protein (MRP) blocker. Cyclosporin A was dissolved in a mixture of ethanol/Cremophor EL (0.015 and 0.27%, w/v, respectively, in buffer A). This mixture was also used as vehicle for control experiments. In some experiments, rIL2 (1 μM) was added in the mucosal and the serosal sides, to study the in vitro interaction between rIL2 and rhodamine 123.

Rhodamine 123 concentrations were measured by a reversed phase-high performance liquid chromatography technique with spectrofluorometric detection. It was separated on a C_8 column (3.9 $\times$ 150 mm, Nova-Pak; Waters, Saint-Quentin-en-Yvelines, France) using a mixture of acetonitrile and 0.05 M sodium phosphate buffer, pH 2.83 (25:75, v/v) as mobile phase. The flow rate was 1 ml/min (RF-10AS pump; Shimadzu, Kyoto, Japan). Under these conditions, retention time of rhodamine 123 was 10 min. Detection was set at 500 and 525 nm for excitation and emission, respectively, using an RF-551 spectrofluorometer (Shimadzu). Ten microliters of each sample was injected in the chromatographic system. The interday coefficients of variation in the studied concentration range (5–200 nM) were below 10%. The limit of quantification was 1 nM.

Semiquantitative Determination of Pgp Expression by Western Blot. After laparotomy, the intestines of mice were quickly removed and washed with ice-cold isotonic saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. Mucosae were scraped on ice with a slide glass and homogenized using a glass Teflon potter (20 strokes) in a buffer containing 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 1 mM PMSF, and 0.1 U/ml aprotinin. The homogenates were centrifuged 10 min at 3,000*g*, and the supernatant was again centrifuged for 30 min at 15,000*g*. The pellets containing the crude membranes were resuspended in 0.5 ml of a buffer containing 50 mM mannitol, 50 mM Tris pH 7.4, 1 mM PMSF, and 0.1 U/ml aprotinin and stored at −80°C until use. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA; Sigma-Aldrich) as standard.

Crude membrane suspensions were diluted to a final protein concentration of 0.5 μg/μl in 1% Triton and placed at 4°C during 30 min for solubilization of Pgp. A volume of 10 μl of loading buffer (240 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.1% bromphenol blue, and 20% 2-mercaptoethanol) was added to 40 μl of each solubilized sample. Ten micrograms of protein was separated onto a 7.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline containing 0.05% Tween 20 (PBST) and 3% skim dried milk for 2 h at room temperature and washed two times for 5 min in PBST. The primary monoclonal antibody C219 (DAKO, Glostrup, Denmark), diluted 200-fold in PBST-1% BSA, was incubated overnight at 4°C. After washes in PBST buffer, secondary polyclonal antibody, mouse anti-IgG peroxidase conjugate (Biosys, France) was diluted 800-fold in PBST-1% BSA and incubated at room temperature for 2 h. Pgp protein in crude membranes was detected by chemiluminescence using enhanced chemiluminescence Western blot reagents (PerkinElmer Life Sciences, Boston, MA). A charge-coupled device camera was used to take the bands. Their intensities were estimated with Digital Analysis ID software (Eastman Kodak, Rochester, NY).

Because IL2 is a Pgp substrate, it could interact with Pgp, masking adequate revelation by C219 in Western blot analysis. Mouse intestinal mucosa (pool of two mouse intestines) was scrapped, and rIL2 was added to the samples at 0 (controls), 30, 300, 3,000, and

30,000 ng/ml in buffer before homogenization. Crude membranes were prepared as described above. Pgp expression of the five samples was measured by Western blot.

Semiquantitative Determination of mdr1a mRNA. After laparotomy, intestines of mice were removed and washed with sterile ice-cold isotonic saline. Mucosae were scraped with a slide glass, on ice, in sterile conditions.

Total RNA was extracted from mucosa using Trizol (Sigma-Aldrich). RT of 2.5 μg of total RNA using 0.25 μg of Oligo(dT)$_{12-18}$ primer was performed for 50 min at 42°C using Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). The technique of Murphy was applied to quantify the expression of specific mRNAs in each sample (Murphy et al., 1990). The polymerase chain reaction (PCR) was found to be both sensitive and quantitative during the exponential range of the reaction. To reach these experimental conditions, serial dilutions of the RNA RT products (0.702–62.5 ng) were amplified by PCR during 35 cycles. Finally, 7.8 ng of the RNA RT products of each sample was amplified by semiquantitative PCR. PCR amplification using specific primers for mdr1a (forward-5′AGCATCTGTGAACCACAT3′; reverse-5′GTTGCTGTTCTACCGCTGG3′) and for β-actin (used as housekeeping gene) as described previously (Damon et al., 1996) was performed in the presence of *Taq*DNA polymerase (Roche Applied Science). For mdr1a and β-actin amplifications, a 5-min incubation at 94°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 60 s. The final extension was at 72°C for 7 min. PCR products were separated on a 1.2% agarose gel stained with 2 μg/ml ethidium bromide, to visualize the DNA bands.

In Vivo Digoxin Pharmacokinetic Studies. Mice received digoxin (5 μg/0.1 ml Digoxin Nativelle solution diluted up to 1 μg/0.1 ml with distilled water) by gavage (33.3 μg/kg of body weight). Mice were killed by decapitation at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 h after digoxin administration. Six animals per each time point were used in both control and rIL2-pretreated groups.

Blood was collected on heparinized polypropylene tubes and centrifuged at 4000*g* for 20 min at 4°C. Plasma was stored at −20°C until digoxin analysis. Plasma digoxin concentration was measured using an automated microparticle enzyme immunoassay (Abbott, Saint-Remy-sur-Avre, France).

In Vivo Saquinavir Pharmacokinetic Studies. For oral administration, the content of Invirase capsules was mixed with 0.2% (carboxymethyl)cellulose to obtain a saquinavir suspension of 8 mg/ml. Saquinavir was administered at 100 mg/kg of body weight by gavage. Mice were killed by decapitation at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, and 180 min after saquinavir administration.

For intravenous administration, saquinavir was dissolved in a 7.5% ethanol/7.5% Cremophor EL saline solution to obtain a concentration of 1.5 g/l. Saquinavir was administered at 7.5 mg/kg of body weight by injection in the tail vein. Mice were killed by decapitation at 3, 6, 10, 20, 30, 45, 60, 90, 120, 150, and 180 min after saquinavir administration. Six animals per each time point were used in both control and rIL2-pretreated groups, for oral and i.v. studies. Blood was collected on heparinized polypropylene tubes and centrifuged at 4000*g* for 20 min at 4°C. Plasma was stored at −20°C until saquinavir analysis.

Plasma saquinavir was quantified by a reversed phase high-performance liquid chromatography method with a spectrophotometric detection at 240 nm. Separation was made on a C_{18} column (4.6 ×250 mm, 5-μm Hypersil; Hypersil, Les Ulis, France). Mobile phase was a mixture of 0.4% trifluoroacetic acid/distilled water/acetonitrile/diethylamine (10:35:55:0.1 v/v/v/v). pH was adjusted at 6.5 with acetic acid. The flow rate was 1 ml/min.

A volume of 100 μl of borate buffer (0.1 M, pH 9.5) was added to 100 μl of plasma and vortexed during 20 s. Then, a volume of 300 μl of methanol was added and vortexed during 20 s. After centrifugation (10 min at 12,000*g*), an aliquot of 350 μl of supernatant was evaporated to dryness under air. Samples were reconstituted in 100 μl of mobile phase. For the analysis, 20 μl was injected in the chromatographic system.

The interday coefficients of variation in the studied concentration range (0.09–9 mg/l) were below 10%. The limit of quantification was 0.045 mg/l.

Data Analysis. The results are expressed as mean ± S.E.M. Statistical comparisons between control group and rIL2-pretreated group were made using a Student's *t* test. Statistical significance was accepted as $p < 0.05$.

Compartmental methods were used to calculate digoxin and saquinavir pharmacokinetic parameters. The MicroPharm software was used. The area under the plasma drug concentration-time curve (AUC) was calculated by the linear trapezoidal method from 0 to 24 h for digoxin and from 0 to 180 min for saquinavir.

Results

Transport of Rhodamine 123 in Mouse Everted Gut Sac. The transport of rhodamine 123 from the serosal to the mucosal side of the gut was investigated during 180 min. The transport was linear between 20 and 100 min. The curve was no more linear over 100 min, suggesting a decrease in the viability of the everted gut sac. The rate of transport was measured between 20 and 80 min.

The rate of transport was measured in the absence and in the presence of several competitors to test the specificity of rhodamine 123 secretion in this model (see *Materials and Methods*). The transport of rhodamine 123 was significantly decreased by 73% in the presence of 25 μM verapamil and by 76% in the presence of 250 μM verapamil (Fig. 1A). Therefore, the inhibition of rhodamine transport was already maximal with 25 μM verapamil. This verapamil concentration will be used in the next experiments. The transport of rhodamine 123 was significantly decreased by 75% in the presence of 10 μM cyclosporin A and by 79% in the presence of 50 μM cyclosporin A (Fig. 1B). Therefore, the inhibition of rhodamine 123 transport was already maximal with 10 μM cyclosporin A.

The transport was not affected by 25 μM probenecid or by 100 μM probenecid (Fig. 1C). The transport of rhodamine 123 that can be inhibited by Pgp blockers was considered as the difference between the basal transport (without inhibitor) and the residual transport (in the presence of Pgp inhibitors). This Pgp-mediated transport of rhodamine 123 was 0.55 ± 0.04 and 0.52 ± 0.05%/min, respectively, in the presence of 25 μM verapamil and 10 μM cyclosporin A (Fig. 1, A and B). The transport of rhodamine 123 was not affected in the presence of 1 μM rIL2 (Fig. 1D).

Influence of rIL2 Treatment on Rhodamine 123 Transport in Mouse Everted Gut Sac. For everted sacs prepared with intestine from control and rIL2-pretreated mice, rhodamine 123 transport was measured in the absence and in the presence of 25 μM verapamil. The basal transport of rhodamine 123 across mouse intestine was significantly decreased by 32% in rIL2-pretreated group compared with control group (Fig. 2A). In the presence of verapamil, the transport of rhodamine 123 was significantly decreased by 65 and 59% in control and rIL2-pretreated groups, respectively (Fig. 2A). The Pgp-mediated transport of rhodamine 123 was significantly decreased by 37% in rIL2-pretreated group compared with control group (Fig. 2B).

Western Blot Analysis. The monoclonal antibody C219 used for Western blot analyses revealed a double band of 165 and 112 kDa. The intensity was very weak in intestinal crude membranes of rIL2-pretreated mice compared with controls

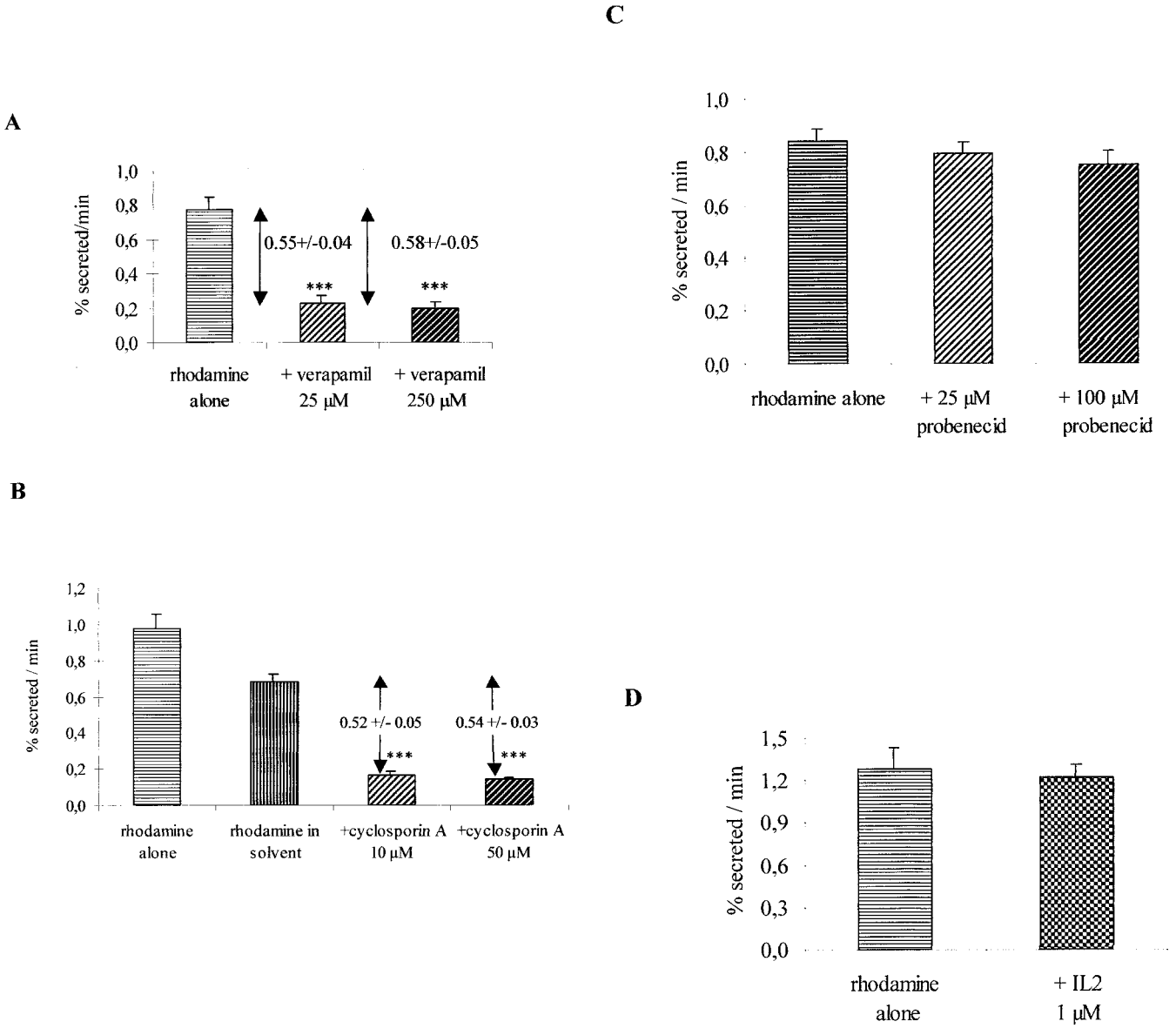

Fig. 1. In vitro transport of rhodamine 123 from serosal-to-mucosal surfaces across mouse everted gut sacs. Rhodamine 123 (25 μM) transport was measured in everted gut sacs in absence or presence of Pgp and MRP blockers. Data are expressed as mean ± S.E.M. ***, significantly different from that without inhibitor ($p < 0.001$). A, in absence and in presence of 25 and 250 μM verapamil ($n = 6$). B, in absence and in presence of cyclosporin A solvent, 10 and 50 μM cyclosporin A ($n = 6$). C, in absence and in presence of 25 and 100 μM probenecid ($n = 7$). D, in absence and in presence of 1 μM rIL2 ($n = 8$).

(Fig. 3A). Another band of 80 kDa also revealed by C219 was similar between both groups. Densitometric analysis of the whole showed a significant decrease of 57% in Pgp protein expression of rIL2-pretreated mice compared with control group (Fig. 3B).

Pgp expression was not modified by the addition of rIL2 in the homogenization buffer for crude membranes preparation (Fig. 4). These results rule out an experimental artifact. The weaker intensity of the bands revealed by Western blot in intestines of mice pretreated with rIL2 really corresponds to a decrease in Pgp protein expression.

Semiquantitative Determination of mdr1a mRNA. The expression of mdr1a and β-actin mRNA showed no difference between control and rIL2-pretreated mice (Fig. 5).

Pharmacokinetics of Digoxin after an Oral Dose, with and without Pretreatment of Mice by rIL2. Plasma levels of digoxin were significantly increased at all time points in mice pretreated with rIL2 compared with controls (Fig. 6). Pharmacokinetic profile of digoxin versus time course fitted in a monocompartmental model. Digoxin AUC was significantly increased by 2.8-fold in rIL2-pretreated mice compared with controls. CL/F and Vd/F were significantly decreased by 60 and 50% in rIL2-pretreated mice compared with controls, respectively. MRT and $t_{1/2\beta}$ were significantly increased by 38 and 30% in rIL2-pretreated mice compared with controls, respectively (Table 1).

Pharmacokinetics of Saquinavir with and without Pretreatment of Mice by rIL2. After oral administration, plasma levels of saquinavir were significantly increased at 30, 45, and 75 min, respectively, by 220, 90, and 160% in rIL2-pretreated mice compared with controls (Fig. 7A). After intravenous administration, plasma levels of saquinavir

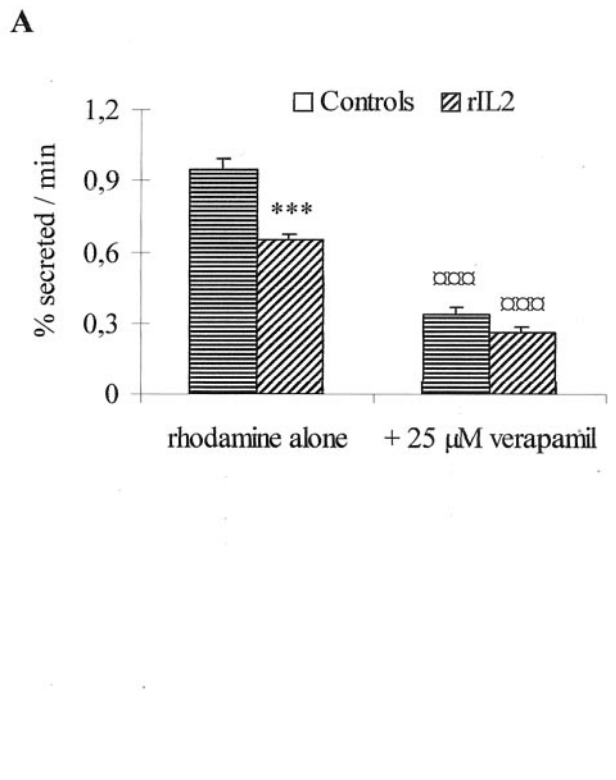

Fig. 2. In vitro transport of rhodamine 123 from serosal-to-mucosal surfaces across everted sacs with intestine of control mice and mice pretreated with rIL2. Mice were treated with 9 MIU/kg rIL2 or 5% glucose i.p. twice daily for 4 days and were sacrificed on day 5. For each mouse, rhodamine 123 (25 μM) transport was measured in everted gut sacs in absence and in presence of 25 μM verapamil. Data are expressed as mean ± S.E.M. (n = 8). A, rhodamine 123 transport in absence and in presence of 25 μM verapamil. ***, significantly different from control mice, $p < 0.001$; ◇◇◇, significantly different from that without verapamil ($p < 0.001$). B, Pgp-mediated transport of rhodamine 123 (difference between transport in absence and in presence of 25 μM verapamil). ***, significantly different from control mice ($p < 0.001$).

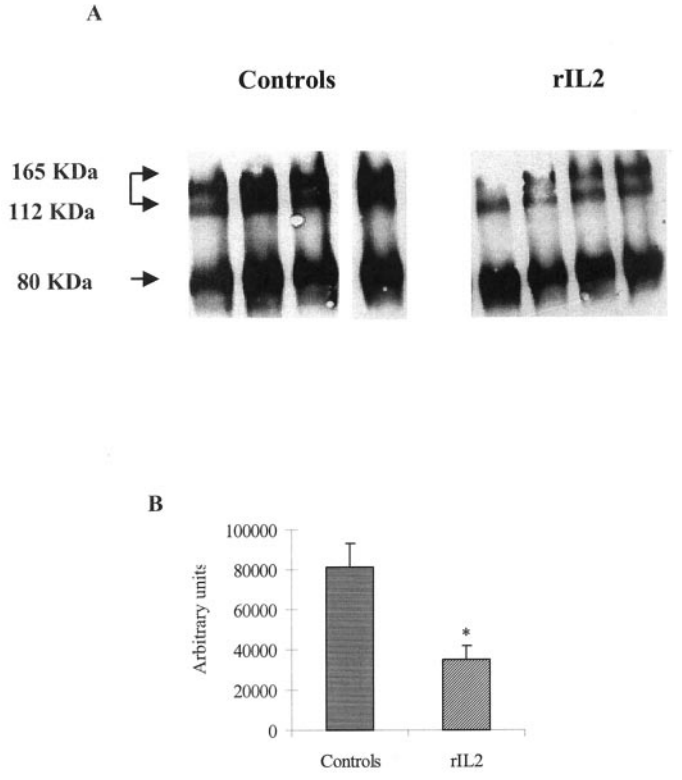

Fig. 3. Expression of Pgp protein in intestine of mice pretreated with rIL2 and control mice. Mice were treated with 9 MIU/kg of rIL2 (n = 8) or with 5% glucose (n = 8) i.p. twice daily for 4 days and were sacrificed on day 5. In each group, mucosae of two mice were pooled to obtain four crude membrane samples by group. Proteins (10 μg) were separated on 7.5% polyacrylamide gel and transferred onto nitrocellulose. The membrane was probed with the monoclonal antibody C219, and the blot was developed with a Western blot chemiluminescence reagent (PerkinElmer Life Sciences). A, immunoblots. B, quantification of the bands; data are expressed as mean ± S.E.M. of arbitrary units (n = 4 pool of two). *, significantly different from control mice ($p < 0.05$).

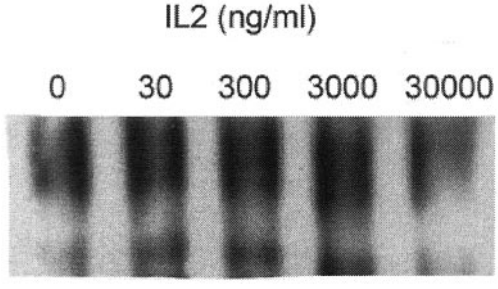

Fig. 4. Expression of Pgp protein after addition of rIL2 in mucosa. Mice intestinal mucosae (pool of two mouse intestine) was scrapped, and rIL2 was added to the samples at 0 (control), 30, 300, 3,000, and 30,000 ng/ml in buffer before homogenization. Crude membrane proteins (10 μg) were separated on 7.5% polyacrylamide gel and transferred onto nitrocellulose. The membrane was probed with the monoclonal antibody C219, and the blot was developed with a Western blot chemiluminescence reagent (PerkinElmer Life Sciences).

were not different between both groups (Fig. 7B). Pharmacokinetic profile of intravenous administered saquinavir fitted in a bicompartmental model. After oral administration, pharmacokinetic profile of saquinavir fitted in a monocompartmental model. rIL2 pretreatment increased the AUC of orally administered saquinavir by 80% and decreased CL/F by 45% (Table 2). For i.v. administration, rIL2 pretreatment did not modify saquinavir AUC, CL, Vd, and MRT (Table 2). Elimination half-life was not affected by rIL2 pretreatment in both oral and intravenous studies (Table 2). The bioavailability of saquinavir was increased by rIL2 pretreatment from 2.5 to 4.5%.

Discussion

Our study demonstrated that rIL2, administered at 9 MIU/kg twice a day intraperitoneally for 4 days in mice, induces a 2-fold decrease in the protein expression of intes-

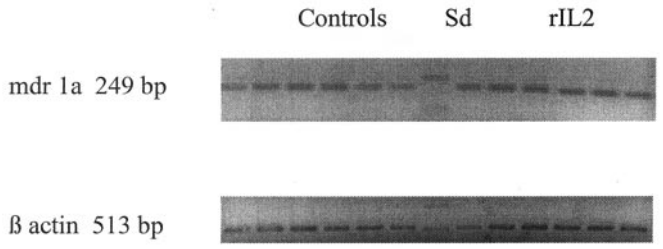

Fig. 5. Expression of mdr1a and β-actin mRNA in intestine by semiquantitative RT-PCR. Mice were treated with 9 MIU/kg of rIL2 or with 5% glucose i.p. twice daily for 4 days and were sacrificed on day 5. Total RNA was extracted from intestinal mucosa using TRIzol. PCR products were separated on a 1.2% agarose gel stained with 2 μg/ml ethidium bromide, to visualize the DNA bands. Sd, PCR standard marker. A, expression of mdr1a mRNA. B, expression of β-actin mRNA.

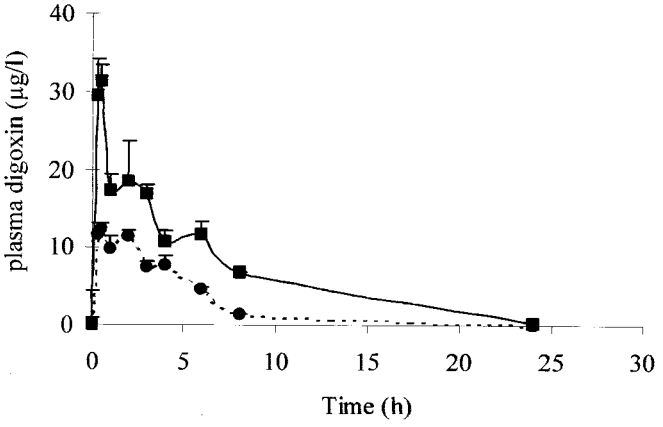

Fig. 6. Digoxin plasma concentration versus time curves after oral administration (33.3 μg/kg). Mice were treated with 9 MIU/kg of rIL2 (solid line; squares) or with 5% glucose (dashed line; circles) i.p. twice daily for 4 days, and pharmacokinetic studies were performed on day 5. Data are expressed as mean ± S.E.M (n = 6).

tinal Pgp. This lower Pgp protein expression led to a 1.6-fold decrease in Pgp activity measured in vitro with everted gut sacs. This decreased Pgp activity was associated with increased digoxin and saquinavir bioavailabilities in mice pretreated with rIL2. This is the first in vivo report where a decrease in intestinal Pgp activity is shown to be induced by a decrease in Pgp protein expression.

In the present study, we have developed a new in vitro model to study the intestinal Pgp function of mice. Rat everted gut sacs have already been used, especially with rhodamine 123 as Pgp substrate (Yumoto et al., 1999; Veau et al., 2001). Conversely, mouse intestine had never been used in the same model until now. The secretion of rhodamine 123 across everted mouse intestine was strongly inhibited by verapamil and cyclosporin A, both validated as Pgp blockers (Hochman et al., 2001; Perloff et al., 2001; Stephens et al., 2001). The residual transport of rhodamine 123 can be attributed to passive pathways or other transporters such as MRP, which is expressed in epithelial cells of mouse intestine (Peng et al., 1999). Moreover, rhodamine 123 has been reported to be transported not only by Pgp but also by MRP in several cancer cell lines (Twentyman et al., 1994). Therefore, we have estimated MRP-mediated transport of rhodamine 123 across mouse intestine with probenecid as a validated MRP inhibitor (Collett et al., 1999; Stephens et al., 2001). Our results showed that the transport of rhodamine 123 was not affected by probenecid proving that this transport is independent of MRP across mouse intestine. This is in agreement with our previous results on rat everted gut sacs and also with a recent work on human colon carcinoma cells (Perloff et al., 2001; Veau et al., 2001). Furthermore, rhodamine 123 is not transported by the organic cation transporter in intestine (van der Sandt et al., 2000; Veau et al., 2001). Therefore, the transport of rhodamine 123 that can be inhibited by verapamil and cyclosporin A can be attributed specifically to Pgp activity.

The Pgp-mediated transport of rhodamine 123 was significantly lower across intestine of mice pretreated with rIL2 compared with control mice. Rhodamine 123 transport (across intestine of control mice) was not affected by the addition of 1 μM rIL2 to the experimental medium. This is in agreement with the study of Drach reporting that rhodamine 123 transport (0.5 μM) is nearly unaffected by the addition of IL2 (20 nM), in human colon carcinoma cells (Drach et al., 1996). Then, the decrease in in vitro Pgp-mediated transport of rhodamine 123 across everted gut sacs of rIL2-pretreated mice can be attributed to the lower Pgp protein expression and not to a direct interaction between rIL2 and rhodamine 123. The decrease in intestinal Pgp protein expression, measured with the specific monoclonal antibody C219 on Western blot, was in agreement with the lower Pgp-mediated transport of rhodamine 123. In contrast, mRNA of *mdr1a*, the gene encoding for Pgp in mouse intestine, was not modified by rIL2 pretreatment. Hypothesis of an experimental artifact masking adequate revelation of Pgp protein in Western blot analysis was ruled out. So, the discrepancy between the Western blot data and the mRNA levels suggests a post-transcriptional modification of the protein by the cytokine. Such a post-transcriptional modification of Pgp level by IL2 is also observed in the study reported by Stein et al. (1996) in human colon carcinoma cells. Therefore, these in vitro results agree with our present in vivo results. Possible post-transcriptional mechanisms have not been investigated in most of the studies reporting modifications of Pgp protein expression. According to our knowledge, an alteration in Pgp translation step has never been demonstrated. According to the literature, only two kinds of modifications could explain a post-transcriptional decrease in Pgp protein expression: one

TABLE 1
Digoxin pharmacokinetic parameters after oral administration (33.3 μg/kg)
Data are expressed as mean ± S.E.M. (n = 6).

	AUC	CL/F	MRT	Vd/F	$t_{1/2\beta}$
	mg min l^{-1}	*10^{-3} l min^{-1} kg^{-1}*	*min*	*l kg^{-1}*	*min*
Controls	3.5 ± 0.5	9.6 ± 0.5	3.4 ± 0.3	202 ± 17	168 ± 15
rIL2	9.7 ± 1.5**	3.8 ± 0.5***	4.7 ± 0.3*	283 ± 19**	218 ± 9*

$t_{1/2\beta}$, elimination half-life.
*/**/*** Significantly different from controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

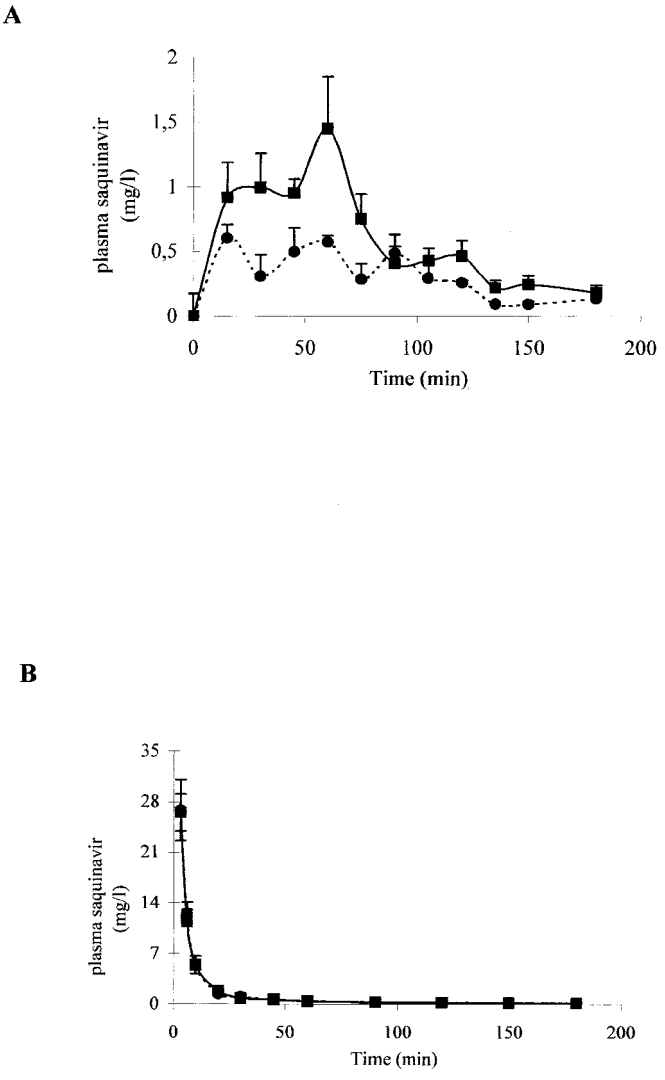

Fig. 7. Saquinavir plasma concentration versus time curves. Mice were treated with 9 MIU/kg of rIL2 (solid line; squares) or with 5% glucose (dashed line; circles) i.p. twice daily for 4 days, and pharmacokinetic studies were performed on day 5. A, after oral administration of saquinavir (100 mg/kg) (n = 6). B, after i.v. administration of saquinavir (7.5 mg/kg) (n = 6). Data are expressed as mean ± S.E.M.

at the level of Pgp glycosylation and the other at the level of Pgp trafficking. First, after synthesis, Pgp needs to be glycosylated to reach the plasma membrane. An alteration in glycosylation involves an increase in the degradation of the native protein, leading to a decrease in its expression (Kramer et al., 1995; Loo and Clarke, 1999; Gribar et al., 2000). Second, a reduction of Pgp movement from a cytoplasmic pool to the cell surface could also explain a down-regulation of Pgp protein expression. This kind of regulation has been reported about Pgp protein overexpression induced by an enhancement of the trafficking of functional Pgp to the plasma membrane (Maitra et al., 2001).

rIL2 pretreatment modified pharmacokinetics of orally administered digoxin as shown by the 2.8-fold increase in plasma AUC in mice pretreated with rIL2 compared with the controls. In mice, the absorption of digoxin as well as the direct intestinal secretion have been shown to be dependent on intestinal Pgp using *mdr1a* knockout mice (Mayer et al., 1996). Digoxin is selectively transported by Pgp across intestine (Stephens et al., 2001). Fifty percent of the initial dose of digoxin is eliminated by the kidney. Kawahara et al. (1999) showed that Pgp contributes to digoxin renal clearance. In the present work, elimination half-life of digoxin was greater in mice pretreated with rIL2 compared with control mice. Renal Pgp protein expression was not affected by rIL2 pretreatment (data not shown). Therefore, the large increase in digoxin AUC can be imputed to the decrease in intestinal Pgp activity induced by rIL2 pretreatment.

rIL2 pretreatment increased plasma AUC of orally administered saquinavir by 80% but did not modify plasma levels of saquinavir administered intravenously. Therefore, bioavailability of saquinavir was increased by 80% (from 2.5 to 4.5%) in mice pretreated with rIL2 compared with control mice. This 1.8-fold increase in saquinavir bioavailability is an important increase considering the relative value but quite small considering the absolute value. Then, absolute bioavailability remains in the lower range (4.5%) after rIL2 pretreatment. Bioavailability of saquinavir is limited by both intestinal and hepatic first-pass metabolism and also by a Pgp-mediated intestinal efflux. The protease inhibitor is mainly eliminated by metabolism via CYP3A. In our study, the total clearance of saquinavir was not affected by rIL2 pretreatment, showing that P450-mediated metabolism of the protease inhibitor was similar between rIL2-pretreated mice and controls. The same range of rIL2 dosage has been reported to reduce total hepatic P450 activity by 20% in mice, and more specifically the isoforms 1A1 and 2B1/2 activities (Cantoni et al., 1995). However, the activity of CYP3A was not specifically measured by Cantoni et al. (1995). According to our i.v. study, activity of CYP3A-mediated metabolism of saquinavir is unaffected by rIL2 pretreatment. These data suggest that rIL2 pretreatment did not affect hepatic first-pass metabolism of orally administered saquinavir. The decrease in intestinal Pgp activity by rIL2 pretreatment did not affect the plasma pharmacokinetic profile of saquinavir after

TABLE 2
Saquinavir pharmacokinetic parameters after oral (100 mg/kg) and i.v. (7.5 mg/kg) administrations
Data are expressed as mean ± S.E.M. (n = 6).

	AUC	CL	CL/F	MRT	Vd	Vd/F	$t_{1/2\beta}$
	mg min l^{-1}	*l* min^{-1} kg^{-1}		*min*	*l* kg^{-1}		*min*
Controls							
7.5 mg/kg i.v.	152 ± 11	0.051 ± 0.004		25 ± 1	5.1 ± 0.2		71 ± 6
100 mg/kg p.o.	55 ± 8		2.2 ± 0.3	74 ± 4		209 ± 81	64 ± 16
rIL2							
7.5 mg/kg i.v.	154 ± 8	0.050 ± 0.003		26 ± 2	5.1 ± 0.3		73 ± 5
100 mg/kg p.o.	98 ± 14*		1.2 ± 0.2*	71 ± 4		92 ± 25	51 ± 9

$t_{1/2\beta}$, elimination half-life.
*/**/*** Significantly different from controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

intravenous administration. This result is in agreement with previous studies in mice depleted in Pgp. After intravenous administration, no difference was observed in saquinavir plasma levels between *mdr1a* (−/−) and *mdr1a* (+/+) mice (Kim et al., 1998; Washington et al., 2000; Huisman et al., 2001). Thus, in our study, the increased bioavailability of saquinavir by rIL2 pretreatment can be explained by an effect on the absorption process, and more specifically by a decrease of the intestinal Pgp-mediated efflux of saquinavir as a consequence of a lower Pgp protein expression. Therefore, a 2-fold decrease in Pgp protein expression led to a small increase in saquinavir bioavailability (Pgp substrate strongly metabolized), showing that in this case, CYP3A-mediated metabolism is the major factor limiting bioavailability.

Furthermore, hypothesis of a direct interaction between rIL2 and saquinavir can be excluded because we studied the direct effect of rIL2 on saquinavir bioavailability in mice. A simultaneous administration of rIL2 (18 MIU/kg i.p.) and saquinavir (100 mg/kg p.o.) did not affect pharmacokinetic profile of the protease inhibitor (data not shown). Therefore, the increase in saquinavir bioavailability can really be imputed to the lower Pgp function induced by the decrease in Pgp protein expression and not to an interaction between rIL2 and saquinavir.

In conclusion, rIL2 treatment led to a decrease in intestinal Pgp protein expression in mice, by a post-transcriptional mechanism. Moreover, rIL2 treatment induced a decrease in Pgp-mediated transport of rhodamine 123 in everted gut sacs. This in vitro model is adequate to measure specifically Pgp activity, especially without interference with MRP-mediated transport. Saquinavir bioavailability was 1.8-fold enhanced after rIL2 treatment. The still low bioavailability of saquinavir despite of the net decrease in Pgp activity outlines the moderate influence of intestinal Pgp on the oral bioavailability of Pgp substrates as strongly metabolized as saquinavir. However, the 2-fold decrease in Pgp protein expression induced a large increase in AUC of orally administered digoxin. These results outline the influence of the decrease in intestinal Pgp protein expression on the oral bioavailability of Pgp substrates poorly metabolized. Moreover, this could have clinical relevance for other weakly metabolized Pgp substrates used in association with rIL2 as anticancer chemotherapy or anti-HIV therapy.

Acknowledgments

We thank Vincent Castagne for the contribution of the dosage of digoxin used in this work.

References

Boyd RA, Stern RH, Stewart BH, Wu X, Reyner EL, Zegarac EA, Randinitis EJ, and Whitfield L (2000) Atorvastatin coadministration may increase digoxin concentrations by inhibition of intestinal P-glycoprotein-mediated secretion. *J Clin Pharmacol* **40:**91–98.

Cantoni L, Carelli M, Ghezzi P, Delgado R, Paggioni R, and Rizzardini M (1995) Mechanisms of interleukin-2-induced depression of hepatic cytochrome P-450 in mice. *Eur J Pharmacol* **292:**257–268.

Collett A, Higgs NB, Sims E, Rowland M, and Warhurst G (1999) Modulation of the permeability of H2 receptor antagonists cimetidine and ranitidine by P-glycoprotein in rat intestine and the human colonic cell line caco-2. *J Pharmacol Exp Ther* **288:**171–178.

Damon M, Fautrel A, Guillouzo A, and Corcos L (1996) Genetic analysis of the phenobarbital regulation of the cytochrome P-450 2b-9 and aldehyde dehydrogenase type 2 mRNAs in mouse liver. *Biochem J* **317:**481–486.

Drach J, Gsur A, Hamilton G, Zhao S, Angerler J, Fiegl M, Zojer N, Raderer M, Haberl I, Andreeff M, and Huber H (1996) Involvement of P-glycoprotein in the transmembrane transport of interleukin 2 (IL-2), IL-4 and interferon-γ in normal human T lymphocytes. *Blood* **88:**1747–1754.

Fitzsimmons ME and Collins JM (1997) Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small-intestinal cytochrome P4503A4. *Drug Metab Dispos* **25:**256–266.

Greiner B, Eichelbaum M, Fritz P, Kreichgauer HP, von Richter O, Zundler J, and Kroemer HK (1999) The role of intestinal P-glycoprotein in the interaction of digoxin and rifampicin. *J Clin Invest* **104:**147–153.

Gribar JJ, Ramachandra M, Hrycyna CA, Dey S, and Ambudkar SV (2000) Functional characterization of glycosylation-deficient human P-glycoprotein using a vaccinia virus expression system. *J Membr Biol* **173:**203–214.

Hochman JH, Chiba M, Yamazaki M, Tang C, and Lin JH (2001) P-glycoprotein-mediated efflux of indinavir metabolites in Caco-2 cells expressing cytochrome P-450 3A4. *J Pharmacol Exp Ther* **298:**323–330.

Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmöller J, Johne A, Cascorbi I, Gerloff T, Roots I, Eichelbaum M, et al. (2000) Functional polymorphism of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc Natl Acad Sci* **97:**3473–3478.

Huisman MT, Smit JW, Wiltshire HR, Hoetelmans MW, Beijnen JH, and Schinkel AH (2001) P-glycoprotein limits oral bioavailability, brain and fetal penetration of saquinavir even with high doses of ritonavir. *Mol Pharmacol* **59:**806–8013.

Kawahara M, Sakata A, Miyashita T, Tamai I, and Tsuji A (1999) Physiological based pharmacokinetics of digoxin in *mdr1a* knockout mice. *J Pharm Sci* **88:**1281–1287.

Kempf DJ, Marsh KC, Kumar G, Rodrigues AD, Denissen JF, McDonald E, Kukulku MJ, Hsu A, Granneman GR, Baroldi PA, et al. (1997) Pharmacokinetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. *Antimicrob Agents Chemother* **41:**654–600.

Kim RB, Fromm MF, Wandel C, Leake B, Wood AJJ, Roden DM, and Wilkinson GR (1998) The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J Clin Invest* **101:**289–294.

Kramer R, Weber TK, Arceci R, Ramchurren N, Kastrinakis WV, Steele G Jr, and Summerhayes IC (1995) Inhibition of N-linked glycosylation of P-glycoprotein by tunicamycin results in a reduced multidrug resistance phenotype. *Br J Cancer* **71:**670–675.

Loo TW and Clarke DM (1999) The human multidrug resistance P-glycoprotein is inactive when its maturation is inhibited: potential for a role in cancer chemotherapy. *FASEB J* **13:**1724–1732.

Lum BL and Gosland MP (1995) MDR expression in normal tissues. Pharmacologic implications for the clinical use of P-glycoprotein inhibitors. *Hematol Oncol Clin N Am* **9:**319–336.

Maitra R, Halpin PA, Karlson KH, Page RL, Paik DY, Leavitt MO, Moyer BD, Stanton BA, and Hamilton JW (2001) Differential effects of mitomycin C and doxorubicin on P-glycoprotein expression. *Biochem J* **355:**617–624.

Mayer U, Wagenaar E, Beijnen JH, Smit JW, Meijer DKF, van Asperen J, Borst P, and Schinkel AH (1996) Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr1a P-glycoprotein. *Br J Clin Pharmacol* **119:**1038–1044.

Murphy LD, Herzog CE, Rudick JB, Fojo AT, and Bates SE (1990) Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. *Biochemistry* **29:**10351–10356.

Noble S and Faulds D (1996) Saquinavir. A review of its pharmacology and clinical potential in the management of HIV infection. *Drugs* **52:**93–112.

Peng KC, Cluzeaud F, Bens M, Duong Van Huyen JP, Wioland MA, Lacave R, and Vandewalle A (1999) Tissue and cell distribution of the multidrug resistance-associated protein (MRP) in mouse intestine and kidney. *J Histochem Cytochem* **47:**757–767.

Perloff MD, Von Moltke LL, Marchand JE, and Greenblatt DJ (2001) Ritonavir induces P-glycoprotein expression, multidrug-resistance-associated protein (MRP1) expression and drug transported-mediated activity in a human intestinal cell line. *J Pharm Sci* **90:**1829–1837.

Piscitelli SC, Vogel S, Figg WD, Raje SR, Forrest A, Metcalf JA, Baseler M, and Falloon J (1998) Alteration in indinavir clearance during interleukin-2 infusions in patients infected with the human immunodeficiency virus. *Pharmacotherapy* **18:**1212–1216.

Sachs MK, Blanchard LM, and Green PJ (1993) Interaction of itraconazole and digoxin. *Clin Infect Dis* **18:**259–260.

Salphati L and Benet LZ (1998) Effects of ketoconazole on digoxin absorption and disposition in rat. *Pharmacology* **56:**308–313.

Schinkel AH, Smit JJ, Van Tellingen O, Beijnen JH, Wagenaar E, Van Deemter L, Mol CAAM, Van der Valk MA, Robanus-Maandag EC, Te Riele HPJ, et al. (1994) Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:**491–502.

Schuetz EG, Beck WT, and Schuetz JD (1996) Modulators and substrates of P-glycoprotein and cytochrome P4503A coordinately up-regulate these proteins in human colon carcinoma cells. *Mol Pharmacol* **49:**311–318.

Stein U, Walther W, and Shoemaker RH (1996) Reversal of multidrug resistance by transduction of cytokine genes into human colon carcinoma cells. *J Natl Cancer Inst* **88:**1383–1392.

Stephens RH, O'Neill CA, Warhurst A, Carlson GL, Rowland M, and Warhurst G (2001) Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human intestinal epithelia. *J Pharmacol Exp Ther* **296:**584–591.

Terao T, Hisanaga E, Sai Y, Tamai I, and Tsuji A (1996) Active secretion of drugs from the small intestinal epithelium in rats by P-glycoprotein functioning as an absorption barrier. *J Pharm Pharmacol* **48:**1083–1089.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, and Willingham MC (1987) Cellular localization of the multi-drug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* **84:**7735–7738.

Twentyman PR, Rhodes T, and Rayner S (1994) A comparison of rhodamine 123 accumulation and efflux in cells with P-glycoprotein-mediated and MRP-associated multidrug resistance phenotypes. *Eur J Cancer* **30A:**1360–1369.

van der Sandt ICJ, Blom-Roosemalen MCM, de Boer AG, and Breimer DD (2000)

Specificity of doxorubicin versus rhodamine-123 in assessing P-glycoprotein functionality in the LLC-PK1, LLC-PK1:MDR1 and Caco-2 cell lines. *Eur J Pharm Sci* **11:**207–214.
Veau C, Leroy C, Banide H, Auchere D, Tardivel S, Farinotti R, and Lacour B (2001) Effect of chronic renal failure on the expression and function of rat intestinal P-glycoprotein in drug excretion. *Nephrol Dial Transplant* **16:**1607–1614.
Wacher VJ, Salphati L, and Benet LZ (1996) Active secretion and enterocystic drug metabolism barriers to drug absorption. *Adv Drug Deliv Rev* **20:**99–112.
Wacher VJ, Wu CY, and Benet LZ (1995) Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol Carcinog* **13:**129–134.
Washington CB, Wiltshire HR, Man M, Moy T, Harris SR, Worth E, Weigl P, Liang Z, Hall D, Marriott L, et al. (2000) The disposition of saquinavir in normal and P-glycoprotein deficient mice, rats and in cultured cells. *Drug Metab Dispos* **28:** 1058–1062.
Yumoto R, Murakami T, Nakamoto Y, Hasegawa R, Nagai J, and Takano M (1999) Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P450 3A-related compounds. *J Pharmacol Exp Ther* **289:**149–155.

Address correspondence to: Prof. R. Farinotti, Unité Propre de Recherche de l'Enseignement Supérieur 2706, Faculté de Pharmacie, 5 rue J. B. Clément, 92296 Châtenay-Malabry cedex, France. E-mail: robert.farinotti@cep.u-psud.fr