Pharmacokinetics and Interactions of a Novel CCR5 Receptor Antagonist with Ritonavir in Rats and Monkeys: Role of CYP3A and P-glycoprotein

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Running title: CYP3A and P-gp in MRK-1 interactions with ritonavir

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List of Abbreviations:

AUC: area under the plasma concentration-time curve; CCR5: Chemokine receptor 5; CL_{blood}: Systemic blood clearance; CL_{int}: Intrinsic clearance; C_{max}: Maximal plasma concentration; CL_{p}: Systemic plasma clearance; CYP: cytochrome P-450; K_a: First-order absorption rate constant; K_{el}: First-order elimination rate constant; MS: mass-spectrometry; LC-MS/MS: Liquid chromatography tandem mass spectrometry; P-gp: P-glycoprotein; T_{max}: Time to reach maximal plasma concentration;
Abstract

The mechanisms of pharmacokinetic interactions of a novel anti-HIV-1 CCR5 receptor antagonist (MRK-1) with ritonavir were evaluated in rats and monkeys. MRK-1 was a good substrate for the human MDR1 and mouse Mdr1a transporters and was metabolized by CYP3A isozymes in rat, monkey and human liver microsomes. Both the in vitro MDR1-mediated transport and oxidative metabolism of MRK-1 were inhibited by ritonavir. Although the systemic pharmacokinetics of MRK-1 in rats and monkeys were linear, the oral bioavailability increased with increase in dose from 2- to 10-mg/kg. The systemic plasma AUC of MRK-1 was increased 4-6 fold when a 2- or a 10-mg/kg dose was orally co-administered with 10-mg/kg ritonavir. Further pharmacokinetic studies in rats indicated that P-glycoprotein (P-gp) inhibition by ritonavir increased the intestinal absorption of 2-mg/kg MRK-1 maximally by ~30-40%, and a major component of the interaction likely resulted from its reduced systemic clearance via the inhibition of CYP3A isozymes. Oral co-administration of quinidine (10- and 30-mg/kg) increased both the extent and the first-order rate of absorption of MRK-1 (2-mg/kg) by ~40-50% and ~100-300%, respectively, in rats, thus further substantiating the role of P-gp in modulating the intestinal absorption of MRK-1 in this species. At the 10-mg/kg MRK-1 dose, however, the entire increase in its AUC upon co-administration with ritonavir or quinidine could be attributed to a reduced systemic clearance and no effects on intestinal absorption were apparent. In contrast to rats, the effects of P-gp in determining the intestinal absorption of MRK-1 appeared less significant in rhesus monkeys at either dose.
The CCR5 chemokine receptor is expressed on both monocytes and T-lymphocytes and is believed to play a pivotal role in the pathogenesis of the human immunodeficiency virus (HIV-1) infection. It has been suggested that the entry of HIV-1 into the host cell is facilitated by the interaction of the viral envelope glycoproteins gp120 and gp41 with the host cell CD4 and then either the chemokine receptor CCR5 or CXCR4 (Deng et al., 1996; Dragic et al., 1996). The macrophage-tropic or R5 variants of HIV-1 utilize CCR5 for entry and are predominant during the early asymptomatic stages of infection, while T cell line-tropic or X4 variants can use CCR5 or CXCR4 and appear later in ~50% of patients during persistent infection concomitant with a catastrophic decline in CD4+ T-cell numbers and the development of clinical AIDS (Connor et al., 1997). Human genetic evidence supports CCR5 as a potentially attractive antiviral target. A 32-base-pair deletion in the CCR5 coding region (CCR5Δ32) generates a non-functional receptor and homozygosity for CCR5Δ32 confers resistance to HIV-1 infection in populations at high risk for exposure but does not manifest any adverse health effect (Liu et al., 1996). Studies of infected humans heterozygous for CCR5Δ32 have shown that the genotype is associated with delayed progression to clinical AIDS (Balfe et al., 1998). A number of CCR5 receptor antagonists with antiviral activity have been identified and are in various stages of clinical development (Moore and Stevenson, 2000; Eckert and Kim, 2001; Finke et al., 2002).

Over the past several years, multi-drug therapy has shown considerable advantage over the use of a single drug in the management of HIV infection (Torres et al., 1997; Palella et al., 1998). This has been propelled by the need to delay the development of resistance and avoid dose-limiting adverse effects with a single agent. Currently, a triple or a quadruple therapy with two nucleoside analogues, plus one or two protease inhibitors, is considered essential for optimal
efficacy and to avoid rapid development of viral resistance (Barry et al., 1997 & 1999). Although the availability of a CCR5 antagonist may offer another powerful pharmacological intervention for the management of HIV infection, it is almost certain that a combination therapy would be required in order to achieve reasonable reductions in disease progression and to circumvent rapid development of resistance.

Ritonavir is one of several HIV-protease inhibitors (ritonavir, indinavir, saquinavir, nelfinavir, amprenavir and lopinavir) approved for the management of HIV infection in the US. Protease inhibitors, especially ritonavir, have potent inhibitory effects on drug metabolizing enzymes such as CYP3A4, 2C9, 2C19 and 2D6 (Eagling et al., 1997; von Moltke et al., 1998). Ritonavir, when given in combination with other protease inhibitors, serves to enhance their pharmacokinetics by providing an increased plasma concentration and prolonged drug residence in the circulation (Hsu et al., 1998; Barry et al., 1999). Thus, potent antiviral effects can be achieved with lower doses of each protease inhibitor and with a less frequent dosing regimen. This has become an important therapeutic strategy for the pharmacotherapy of HIV. In addition to their inhibitory effects on CYP enzymes, protease inhibitors including ritonavir are also good substrates for the human multi-drug resistance protein MDR1 or P-gp (Kim et al., 1998; Lee et al., 1998; Alsenz et al., 1998). Thus, these compounds have the ability to modulate and/or inhibit P-gp mediated transport, with ritonavir being the most potent in this regard (Gutmann et al., 1999; Profit et al., 1999).

In order to effectively manage and utilize drug-drug interactions towards a therapeutic benefit during the management of HIV infection in the clinic, a thorough understanding of the potential
biochemical mechanisms responsible for these interactions is required. Thus, we undertook a series of pharmacokinetic and interaction studies with a novel investigational CCR5 receptor antagonist, 2-(R)-[N-Methyl-N-(1-(R)-3-(S)-((4-(3-benzyl-1-ethyl-(1H)-pyrazol-5-yl)piperidin-1-yl)methyl)-4-(S)-(3-fluorophenyl)cyclopent-1-yl)amino]-3-methylbutanoic acid (MRK-1, Finke et al., 2002; Figure 1), alone or in combination with ritonavir in rats and monkeys. Our aim was to elucidate the relative significance of P-gp-mediated modulation of intestinal absorption and CYP3A-catalyzed oxidative metabolism in the pharmacokinetic interactions of MRK-1 with ritonavir in rats and monkeys.
Methods

Materials

MRK-1 (2-(R)-[N-Methyl-N-(1-(R)-3-(S)-((4-(3-benzyl-1-ethyl-(1H)-pyrazol-5-yl)piperidin-1-yl)methyl)-4-(S)-(3-fluorophenyl)cyclopent-1-yl)amino]-3-methylbutanoic acid) was synthesized within the Department of Medicinal Chemistry, Merck Research Labs, Rahway, NJ. $[^{3}H]$MRK-1 (specific activity 16.6 mCi/mg) and $[^{14}C]$MRK-1 (specific activity 42.98 µCi/mg) were synthesized by the Labeled Compound Synthesis Group, Department of Drug Metabolism, Merck Research Laboratories, Rahway, NJ. Quinidine gluconate was purchased from Sigma Chemical Co. (St. Louis, MO). Ritonavir was obtained as the commercially available Norvir® solution from Abbott Laboratories (Abbott Park, IL). Polyclonal rabbit antiserum against rat cytochrome P-450 (CYP) 3A2, 2C11 and the corresponding control antiserum (non-immune serum) were purchased from Gentest Corporation (Woburn, MA). Monoclonal antibodies against human CYP3A4, 2D6 and 2C9 were raised in-house in mice following immunization with individual recombinant isozymes, as described previously (Mei et al., 1999). Microsomes containing individual recombinant human CYP isozymes were also prepared in-house from Sf21 insect cells infected with recombinant baculoviruses encoding individual CYP cDNA’s (Mei et al., 1999). All other chemicals were purchased from Sigma Chemical Co. and were of reagent grade.

Identification of Cytochrome P-450 Isozymes Responsible for MRK-1 Metabolism
[^3H]MRK-1 was incubated at 37°C with microsomes prepared from baculovirus-infected cells containing individually expressed CYP isozymes and cytochrome P-450 reductase. Each incubation contained 10-µM[^3H]MRK-1, 500 pmol/mL CYP protein, an NADPH-regenerating system consisting of glucose-6-phosphate (10-mM), NADP⁺ (2-mM) and glucose-6-phosphate dehydrogenase (2.8 units/mL), and magnesium chloride (10-mM) in 100-mM potassium phosphate buffer (pH 7.4). Incubations were carried out for 60-min, after which time the reaction was halted by the addition of an equal volume of acetonitrile. Following centrifugation, the supernatant was analyzed by HPLC with an on-line radioactivity detector.

Further confirmation of the CYP isoform(s) responsible for the in vitro metabolism of[^3H]MRK-1 (10-µM) in human liver microsomes was obtained by incubating the compound in the presence of monoclonal antibodies against CYP2C9, CYP2D6 and CYP3A4, and also with the cytochrome P-450 isoform-specific inhibitors including sulfaphenazole (CYP2C9), tranylcypromine (CYP2C19), quinidine (CYP2D6), and ketoconazole and troleandomycin (CYP3A4). Effect of the above monoclonal antibodies on MRK-1 metabolism was also examined in male rhesus monkey liver microsomes. In addition, male rat liver microsomes were incubated with polyclonal antibodies against CYP2C11 and 3A2 to examine the role of these CYPs in MRK-1 metabolism in the rat. Each incubation contained 1-mg/mL microsomal protein and 25-µL/mL of the antibody preparation along with the above described buffer and NADPH-regenerating system. The disappearance of MRK-1 from the incubation was determined by LC-MS/MS. Metabolite profiles in these incubations were also examined by radiochromatography.
The potential of ritonavir to inhibit the metabolism of MRK-1 was also examined in rat, monkey and human liver microsomes. MRK-1 (10-μM) was incubated, as above, with liver microsomes from the three species in the absence and presence of varying concentrations of ritonavir for 15-min. Preliminary studies indicated that metabolism was linear for the duration of the incubation. At the end of the incubation, the reaction was stopped by the addition of an equal volume of acetonitrile. Samples were spun in a centrifuge and the supernatant was analyzed for MRK-1 concentrations using LC-MS/MS. The rates of MRK-1 metabolism in the presence of ritonavir were calculated relative to the control and the data were fit to the equation, % Activity Remaining = 100*IC50/[IC50+I] in order to determine the ritonavir concentration required for 50% inhibition of MRK-1 metabolism (IC50) under these conditions. ‘I’ represents the inhibitor concentration.

Transepithelial Transport of MRK-1 Across Monolayers of Cell Lines Transfected with Human MDR1 and Mouse Mdr1a Transporter and the Effect of Ritonavir

Human MDR1 transfectants (L-MDR1), mouse Mdr1a transfectants (L-Mdr1a) and their parental pig kidney epithelial cell line (LLC-PK1) were kindly provided by Dr. Alfred H. Schinkel (The Netherlands Cancer Institute) and used under a license agreement. Cells were cultured in Medium 199 (GIBCOBRL) supplemented with 2-mM L-glutamine, penicillin (50 units/mL), streptomycin (50 μg/mL) and 10% (v/v) of FCS (GIBCOBRL) (Schinkel et al., 1995). For L-MDR1 and L-Mdr1a, cells were maintained in the continuous presence of vincristine (640-nM) (Schinkel et al., 1995). Confluent monolayers were subcultured every 3-4
days by treatment with 0.25% trypsin, and 1-mM EDTA in Ca\(^{2+}\)-and Mg\(^{2+}\)-free Hank’s balanced salt solution. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air.

Transepithelial transport studies were carried out as described by Schinkel et al. (1995), with minor modifications. L-MDR1, L-Mdr1a and LLC-PK1 cells were plated at a density of 4 x 10\(^5\) cells/12-mm well on porous (3-µm) polycarbonate membrane filters (Transwell\textsuperscript{TM}; Costar Corp., Cambridge, MA). Cells were supplemented with fresh media every 2 days and used in the transport studies on the fourth day after plating. Transepithelial resistance was measured in each well using a Millicell ohmmeter (model ERS; Millipore Corp., Bedford, MA); wells registering a resistance of 300Ω or greater, after correcting for the resistance obtained in control blank wells, were used in the transport experiments.

About 1-2 hr before the start of the transport experiments, the medium in each compartment was replaced with fresh transport medium. The transport experiment was then initiated (t=0) by replacing the medium in each compartment with 700-µL of transport medium with (donor compartment) and without (receiver compartment) the radiolabeled substrate ([\(^{14}\)C]MRK-1, 10-µM, 0.5-µCi/mL). Directional transport of vinblastine ([\(^{3}\)H]Vinblastine, 10-µM, 0.5-µCi/mL) was examined in parallel as a positive control for P-gp activity. After 0.5, 1 and 2-hr, 50-µL aliquots were taken from the receiver compartment and replaced with fresh transport medium. Samples were placed in scintillation vials containing 5-mL of scintillation cocktail (Ultima-Flo M, Packard Instrument Company, Meriden, CT), and total radioactivity was measured by liquid scintillation counting. The data were calculated either as fraction of the total added radioactivity
that appeared in the receiver compartment or as the total amount transported as a function of time.

In order to examine the effect of ritonavir on MRK-1 transport in L-MDR1 cell monolayers, varying amounts of ritonavir (in ethanol at a final concentration of 2%) were added to both the donor and receiver compartments to provide final concentrations of 0, 10, 25, 50, and 75-µM. The effect of cyclosporin A (10-µM) on MRK-1 and vinblastine transport was examined also as a positive marker for P-gp inhibition. Directional transport was measured in three individual cell cultures on three separate days and in triplicate during each experiment. The data are presented as the mean ± S.D.

The transport of MRK-1 in both apical-to-basolateral (A-to-B) or basolateral-to-apical (B-to-A) direction was linear during the 2-hr experimental period. Thus, the average rate of MRK-1 transport in each experiment was calculated from the slope of total amount transported vs. time plot. The concentration of ritonavir resulting in 50% inhibition of P-gp mediated MRK-1 transport (IC50) was calculated as described below. The rate of A-to-B transport of MRK-1 in LLC-PK1 and L-MDR1 cells was calculated as described above from the slope of total amount transported vs. time plot. The P-gp mediated transport rate of MRK-1 was obtained then by subtracting the A-to-B transport rate in L-MDR1 cell lines (both in the presence and absence of various concentrations of ritonavir and cyclosporin A) from that in LLC-PK1 cells. The data on % P-gp mediated MRK-1 transport activity remaining in the presence of various concentrations of ritonavir relative to the control were then fit to the equation of the form below in order to obtain IC50 of ritonavir.
% P-gp Transport Activity Remaining = 100*IC_{50}/(Ritonavir Concentration + IC_{50})

**Effect of P-glycoprotein Inhibitors on MRK-1 Absorption: Studies in the Isolated Rat Mesenteric Intestinal Loop Preparation**

The absorption of MRK-1 was examined in isolated rat intestinal loop preparations in order to determine whether MRK-1 was a substrate for efflux transporters in the rat intestine and if its absorption could be modulated by P-gp inhibitors. The detailed surgical procedures were similar to those described elsewhere (Lin et al., 1996). Briefly, rats (n=3/group) were anesthetized with pentobarbital (40-mg/kg i.p.) and a cannula was inserted into the femoral vein. A 10-cm segment of the proximal jejunum was then isolated and both its ends were ligated with a suture. The mesenteric vein draining this intestinal segment was cannulated. All mesenteric venous blood draining from the loop was collected via this mesenteric venous cannula at 10-min intervals. The sampled blood was simultaneously replaced with fresh blood from a donor rat by infusion via the femoral vein at approximately the same rate that blood drained from the mesenteric venous cannula (0.1–0.15 mL/min). Blood samples were collected every 10-min for up to 60-min after the injection of a 0.1 mg dose of MRK-1 (in 0.15 mL PEG400:ethanol:water, 20:20:60 by volume) into the intestinal loop. In order to examine the effect of P-gp modulators on MRK-1 absorption, either a 0.1 mg dose of ritonavir or verapamil was included along with MRK-1 in the dosing solution. Plasma was harvested from the collected mesenteric blood samples by centrifugation and analyzed for MRK-1 concentrations using LC-MS/MS.
Plasma Protein Binding and Blood-to-Plasma Partitioning of MRK-1

In vitro plasma protein binding of [³H]MRK-1 in Sprague-Dawley rats, rhesus monkeys and human male volunteers was determined at 0.01, 0.1, 1 and 10-µg/mL using an established ultracentrifugation method. All plasma used in these experiments was freshly obtained. Blood-to-plasma partitioning was determined by adding known concentrations of [³H]MRK-1 to whole blood and subsequently determining the radioactive content of the plasma following centrifugation.

Pharmacokinetics in Rats and Monkeys

All animal procedures were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Rats and monkeys were housed in temperature and humidity-controlled rooms with a 12-hr light/dark cycle.

Cannulas were implanted in femoral artery and vein of male Sprague-Dawley rats (250-300 g, n=3 or 4/ group) and animals were allowed to recover from surgery for at least one day before experimentation. Similarly, male adult rhesus monkeys (Macaca mulatta, n=4/group) of 5-7 years of age were surgically prepared by placing catheters either into the saphenous vein via percutaneous venipuncture, or by surgically placing indwelling catheters into the femoral vein and connecting them to a subcutaneous vascular access port. Monkeys were transferred to restraint chairs on the day of experiment for dosing and blood collection. Rats and monkeys
were fasted overnight before drug administration, whereas access to water was provided ad libitum. Food was restored after the collection of 4-hr blood samples.

Intravenous dosing solutions of MRK-1 were prepared in a PEG400:EtOH:Water (2:2:6, by volume) vehicle. The compound was administered as an i.v. bolus via the femoral vein (or saphenous vein in case of monkeys) at 0.5 and 2-mg/kg doses at a dose volume of 1- (rats) or 0.2-mL/kg (monkeys). Oral dosing solutions of MRK-1 were prepared as a suspension in 0.9% NaCl and the doses were 2- and 10-mg/kg in a dosing volume of 1- (monkeys) or 5-mL/kg (rats). Different groups of rats were used for oral and i.v. administration experiments. However, a randomized two-way cross over design was used for the oral and i.v. administration experiments in monkeys.

Effect of Ritonavir Oral Co-administration on the Pharmacokinetics of MRK-1 in Rats and Monkeys

Separate groups of rats were surgically prepared as above to examine the effect of ritonavir oral co-administration on the pharmacokinetics of MRK-1. However, the same set of rhesus monkeys that were used in the previous pharmacokinetic experiments (vide supra) were used for these studies. Appropriate doses of ritonavir were administered as the commercially available Norvir® solution. The oral doses and dosing volumes of MRK-1 were the same as described above for the pharmacokinetic studies. Animals were administered the ritonavir dose via oral gavage followed immediately by the MRK-1 suspension via the same route.
Effect of Quinidine Oral Co-administration on the Oral Pharmacokinetics of MRK-1 in Rats

Rats (n=3/group) were surgically prepared as above. Formulations were prepared by dissolving appropriate amounts of MRK-1 and quinidine (quinidine gluconate, Sigma, St. Louis, MO) in a EtOH:PEG400:Water (2:2:6, by volume) vehicle. The dosing volume was 5-mL/kg in each case. MRK-1 was administered either alone (at 2- and 10-mg/kg doses) or in combination with 10- and 30-mg/kg quinidine.

Effect of Oral Ritonavir and Quinidine on the Systemic Pharmacokinetics of MRK-1 in Rats

Quinidine formulations were prepared at appropriate concentrations, as described above, in EtOH:PEG400:Water (2:2:6, by volume). Norvir® solution was used for ritonavir doses. A 0.5 mg/mL solution of MRK-1 was prepared in the EtOH:PEG400:Water (2:2:6, by volume) vehicle. Rats (n=3/group) were administered either vehicle, ritonavir (10-mg/kg), or quinidine (30-mg/kg) doses 

via oral gavage 30-min before the administration of a 0.5-mg/kg i.v. bolus dose of MRK-1; the 30-min time-point corresponds to plasma concentrations of ritonavir that are near maximal (Cmax) with this dosing regimen (data not shown).

In all pharmacokinetic and interaction studies, blood samples (250-μL for rats and 1-mL for monkeys) were collected at predetermined time points up to 24-hr after drug administration.
Plasma was obtained by centrifugation of the blood and stored at $-20\,^{\circ}\mathrm{C}$ until LC-MS/MS analysis.

**LC-MS/MS Analysis**

Plasma samples were extracted by a solid phase extraction procedure that utilized Waters Oasis™ 96-well extraction plates. Briefly, the 96-well plates were equilibrated, successively, in two steps with 1 mL each of methanol and water. An aliquot of 1M phosphoric acid (0.5-mL) was added to each sample well. Appropriate volumes of calibration curve standard solutions, quality control samples (prepared in control rat or monkey plasma), and plasma samples (0.1 mL) were pipetted into the predetermined sample wells. Control plasma (0.1-mL) was included in each of the calibration curve samples. The internal standard (a close analog of MRK-1, 50 ng) was added to all wells and the contents of each well were thoroughly mixed. The plate was eluted slowly under vacuum until the wells were dry and each sample well was then washed with 0.5-mL of distilled water. The sample wells were eluted with 300-µL of acetonitrile:distilled water mixture (90:10, v/v) into a 96-well collection plate and analyzed using LC-MS/MS. Chromatography was performed on an ABZ+ column (100 mm x 2.1 mm, 5 µm, Supelco) and an HPLC system consisting of Perkin Elmer Series 200 Micro Pumps and autosampler using a gradient mobile phase of acetonitrile, methanol and 1-mM ammonium acetate. The HPLC flow rate was 0.35 mL/min. Detection of the analyte and internal standard was performed using a Sciex API 3000 mass spectrometer in the positive ion mode using the Turbo-Ion Spray source at 400°C. Mass transitions (m/z) monitored were 575 $\rightarrow$ 444 for MRK-1 and 547 $\rightarrow$ 282 for the internal standard. Triplicate calibration curves were constructed by plotting peak area ratio of
the analyte to internal standard against the analyte concentration. The concentrations of MRK-1 in plasma samples were determined by comparing the analyte to internal standard peak area ratios against the calibration curve. Calibration curves for MRK-1 were constructed at a concentration range of 1-1000 ng/mL and the data were fitted to a power model of the form $y = ax^b$. The variability and bias of the LC-MS/MS assay for MRK-1 at all quality control (QC) levels was <15%.

**Pharmacokinetic Analyses**

Pharmacokinetic parameters of MRK-1 were calculated by standard pharmacokinetic approaches (Gibaldi and Perrier, 1982). The AUC up to the last sampling point was calculated by the linear trapezoidal rule. Extrapolation to infinity was performed by the factor $C_{\text{last}}/\lambda_z$, where $C_{\text{last}}$ is the plasma concentration at the last sampling time and $\lambda_z$ is the terminal elimination rate constant.

For determination of the first-order absorption and elimination rate constants in MRK-1-quinidine interaction studies, the plasma concentration-time data were fitted to a 1-compartment model with first-order absorption and elimination.

The intrinsic clearance ($CL_{\text{int}}$) of MRK-1 was calculated from the *i.v.* bolus clearance data, assuming a well-stirred model of hepatic clearance and using the following equations:

$$CL_{\text{blood}} = \frac{Q_H \times CL_{\text{int}}}{Q_H + CL_{\text{int}}} \quad \text{or} \quad CL_{\text{int}} = \frac{Q_H \times CL_{\text{blood}}}{Q_H - CL_{\text{blood}}} \quad (1)$$
where, $Q_H$ and $CL_{\text{blood}}$ refer to hepatic blood flow and systemic blood clearance, respectively.

Also, following oral administration:

$$CL_{\text{int}} = \frac{F_a \cdot \text{Dose}_{\text{oral}}}{AUC_{\text{oral}}} \quad \text{or} \quad \frac{\text{Dose}_{\text{oral}}}{AUC_{\text{oral}}} = \frac{CL_{\text{int}}}{F_a} \quad (2)$$

where, $F_a$, $\text{Dose}_{\text{oral}}$ and $AUC_{\text{oral}}$ refer to the fraction of orally administered dose absorbed into the circulation, total administered oral dose and systemic blood AUC of MRK-1 following an oral dose, respectively.

Assuming linear systemic pharmacokinetics and constant plasma protein binding, the fraction of the orally administered dose that was absorbed following administration of different doses was calculated using the pharmacokinetic data from the $i.v.$ bolus and oral administration experiments and the above two equations. As described in Results, the assumptions of linear systemic pharmacokinetics and constant plasma protein binding were largely true at the plasma concentrations encountered in our studies.
Results

MRK-1 is a Substrate for the CYP3A Isozymes in Rat, Monkey and Human Liver Microsomes

The relative rates of metabolism in liver microsomes from different species followed the rank order monkey ≥ human > rat. Approximately 90, 100 and 60% of the compound was metabolized at the end of a 60-min incubation period when MRK-1 (10-µM) was incubated with rat, monkey and human liver microsomes (1-mg/mL microsomal protein), respectively. The oxidative metabolism of MRK-1 in human and monkey liver microsomal incubations was completely inhibited when microsomes were pre-incubated with monoclonal antibodies against the CYP3A4 isozyme. In contrast, anti-CYP2C8/9 and anti-CYP2D6 antibodies had no significant inhibitory effect on MRK-1 metabolism in either human or monkey liver microsomes. Similarly, anti-rat CYP3A2 antibody inhibited MRK-1 metabolism in rat liver microsomes by ~70%. Consistent with results from antibody studies, incubation of [3H]MRK-1 with microsomes containing individually expressed recombinant human CYPs showed that MRK-1 was metabolized by only CYP3A4; no metabolism was detectable in microsomes containing any of the other human CYP isozymes. In addition, the use of specific chemical inhibitors of human CYP isozymes indicated that the phase I metabolism of MRK-1 in human liver microsomes could be inhibited completely by ketoconazole (a potent reversible inhibitor of CYP3A4) and by >70% by troleandomycin (a mechanism-based inhibitor of CYP3A4). In contrast, the inhibitors of other CYP isozymes such as sulphaphenazole (CYP2C9), quinidine (CYP2D6), and tranylcypromine (CYP2C19) exhibited only minor inhibitory effects (<20% inhibition) on the
metabolism of MRK-1 in human liver microsomes up to a high concentration of 50-µM. Thus, data from experiments with human in vitro systems and from the effect of anti-CYP3A antibodies on MRK-1 metabolism in liver microsomes suggest that the compound was metabolized primarily by the CYP3A isozymes in the three species.

Ritonavir was a potent inhibitor of MRK-1 metabolism in rat, monkey and human liver microsomal incubations, with IC₅₀ values of 0.29, 0.53 and 0.23-µM, respectively.

**MRK-1 is a Substrate for P-glycoprotein**

MRK-1 showed a substantially greater B-to-A than A-to-B transport in monolayers of human MDR1 or mouse Mdr1a-transfected cell lines, while the transport in the two directions was roughly equal in the parental LLC-PK1 cells (Table I). These data suggest that MRK-1 is a good substrate for human MDR1 and mouse Mdr1a transporters. However, the B-to-A/A-to-B transport ratio of MRK-1 was consistently lower than that of the prototypical P-gp substrate vinblastine suggesting that the latter may be a better P-gp substrate than MRK-1. The preferential B-to-A efflux transport of MRK-1 as well as vinblastine in L-MDR1 cells was significantly inhibited by 10-µM cyclosporin A (a known P-gp inhibitor). The P-gp mediated efflux transport of MRK-1 in L-MDR1 cell monolayers was inhibited effectively by ritonavir, with an IC₅₀ of ~15-µM (Figure 2). However, cyclosporin A appeared to be somewhat more potent as an inhibitor of human MDR1 relative to ritonavir (Figure 2).

**Plasma protein binding and blood-to-plasma partitioning of MRK-1**
MRK-1 was bound extensively to plasma proteins in all species, with the average plasma protein binding in rat, monkey and human plasma being 99.6, 99.3 and 99.5%, respectively. The average blood-to-plasma partition ratio of [3H]MRK-1 radioactivity was 0.62, 0.62 and 0.58 in the rat, monkey and human, respectively. Both, MRK-1 plasma protein binding and blood-to-plasma ratio, were independent of concentration between 0.01 – 10 µg/mL.

**Rat Intestinal Loop Studies**

Figure 3 shows the net amount of MRK-1 absorbed from a jejunal segment of the rat intestine during 10-min sampling intervals for up to an hour following the administration of a 0.1-mg dose. Co-administration of verapamil or ritonavir (0.1-mg) with MRK-1 increased the net absorption of the latter compound by ~2-3 and >25 fold, respectively, at each sampling time during the 60-min experimental period.

**Pharmacokinetics of MRK-1 in Rats and Monkeys**

Pharmacokinetic parameters of MRK-1 in rats and monkeys after i.v. and oral dosing are presented in Table II. Plasma concentrations of MRK-1 followed a typical bi-exponential decline following i.v. bolus dosing at 0.5 and 2-mg/kg in both rats and monkeys. The pharmacokinetics of MRK-1 after i.v. bolus administration were linear as the dose was increased from 0.5 to 2-mg/kg in both rats and adult monkeys. The compound exhibited a low-to-moderate plasma clearance in both species. The terminal elimination half-life of the compound
was short (~1-3 hr) in both species. Because of the very high plasma protein binding of MRK-1, the steady-state volume of distribution was also in the low-to-moderate range (~1-2 L/kg). In both rats and adult monkeys, the bioavailability increased non-linearly with an increase in oral dose from 2 to 10-mg/kg; however, the increase in bioavailability in monkeys was statistically non-significant (paired t-test, \( p \geq 0.05 \))

The average fraction of administered oral dose of MRK-1 that was absorbed from the intestine into the circulation in rats and monkeys was estimated using the well-stirred model of hepatic elimination as described in Methods. The average hepatic blood flow values for rats and adult monkeys were assumed to be 65- and 45-mL/min/kg, respectively, for the purpose of this calculation (Davies and Morris, 1993). The data presented in Table III suggest that the fraction absorbed increased with increase in oral dose from 2- to 10-mg/kg in both species. The compound appears to be well absorbed, with the fraction absorbed approaching 80-100% at the 10-mg/kg oral dose in rats as well as monkeys.

Effect of Oral Co-administration of Ritonavir on the Pharmacokinetics of MRK-1 in Rats and Monkeys

As described above, MRK-1 is a P-gp substrate and is metabolized mainly by the CYP3A isozymes. In vitro studies described herein suggest that ritonavir is a potent inhibitor of MRK-1 metabolism as well as its P-gp mediated efflux transport. Thus, MRK-1 pharmacokinetics can be influenced by ritonavir via inhibition of both these proteins. The data on the effect of oral co-administration of ritonavir on the pharmacokinetics of MRK-1 are presented in Figure 4 and
Table IV. Thus, when a 2 or 10-mg/kg dose of MRK-1 was orally co-administered with a 10-mg/kg dose of ritonavir to rats, the systemic plasma AUC of MRK-1 was increased between 4-6 fold as compared to control. The increases in MRK-1 AUC following co-administration with ritonavir were also accompanied by increases of similar magnitude in maximal plasma concentrations (C\text{max}) (Table IV). Interestingly, however, there appeared to be little change in the slope of MRK-1 plasma concentration vs. time profile during the terminal elimination phase (Figure 4). Similar to rats, the systemic plasma AUC of MRK-1 was also increased ~5.5 – 6.0 fold when the compound was orally co-administered with a 10-mg/kg dose of ritonavir to rhesus monkeys. In contrast to rats, however, the maximal plasma concentration of MRK-1 in rhesus monkeys was little changed relative to controls (Table IV). Also, in contrast to rats, the plasma concentrations of MRK-1 in rhesus monkeys were maintained at or near C\text{max} for up to 6-8 hr, when given in combination with ritonavir; thereafter, the concentrations appeared to decline in parallel to those in experiments without ritonavir.

Effect of Oral Co-Administration of Quinidine on the Pharmacokinetics of MRK-1 in Rats

A nonlinear increase in the oral bioavailability of MRK-1 in rats and monkeys raises the possibility of involvement of P-gp-mediated efflux in the absorptive processes of MRK-1 at the intestinal mucosal surface such that at higher doses the saturation of this efflux transport may lead to increased absorption and bioavailability. In order to confirm this possibility, studies were conducted to examine the oral pharmacokinetics of MRK-1 when administered in combination with quinidine, a known inhibitor of P-gp. Quinidine was selected because of its relatively low inhibitory potential towards CYP3A isozymes (Achira et al., 1999) that are predominantly
responsible for MRK-1 metabolism (see above). As shown in Figure 5 and Table V, oral co-administration of quinidine at 10 and 30-mg/kg increased the plasma AUC of MRK-1 following a 2-mg/kg oral dose by ~3 fold, along with ~3.5 – 5 fold increase in plasma C_{max}. There was a dose dependent increase in the first-order absorption rate constant and a decrease in model-estimated T_{max} when MRK-1 was administered in combination with quinidine (Figure 5 and Table V), suggesting a more rapid MRK-1 absorption in the presence of quinidine. In contrast, the first-order elimination rate constant of MRK-1 was not affected upon co-administration with quinidine. In contrast to these data, at the 10-mg/kg MRK-1 dose, co-administration with quinidine (30-mg/kg) increased the plasma AUC of MRK-1 by a somewhat smaller magnitude (~2 fold) relative to control and there was little change in C_{max}, K_{a}, K_{el} or T_{max} of MRK-1 (Table V).

Role of Increased Absorption and Reduced Systemic Elimination of MRK-1 in its Pharmacokinetic Interactions with Ritonavir

A profound increase in the plasma AUC of MRK-1 upon oral co-administration with ritonavir in both rats and monkeys raises the question of the relative significance of increased absorption (resulting from inhibition of P-gp at the intestinal mucosal surface) and reduced systemic clearance of MRK-1 (because of inhibition of CYP3A mediated metabolism) in this interaction. We chose to address this issue by resolving the systemic clearance component of this interaction from the overall interaction. This was achieved by examining the effect of oral ritonavir administration on the systemic clearance of MRK-1, as shown in Table VI. The data presented in Figure 6 and Table VI illustrate that a 10-mg/kg oral dose of ritonavir resulted in ~3 fold
reduction in systemic clearance of MRK-1. Considering the magnitude of MRK-1 clearance in rats and assuming a rat hepatic blood flow of 65 mL/min/kg, this amounts to, on average, a 4.5-fold reduction in the intrinsic clearance of MRK-1, based on the well-stirred model of hepatic clearance (Table VI). Since, according to the well-stirred model, $AUC_{\text{oral}} = F_a \cdot \text{Dose}/CL_{\text{int}}$, therefore a 4.5-fold reduction in $CL_{\text{int}}$ of MRK-1 upon co-administration with ritonavir would result in an increase in its systemic AUC of approximately the same magnitude due solely to its reduced systemic elimination. Since, at a 2-mg/kg MRK-1 dose, the total change in systemic plasma AUC of MRK-1 was ~6 fold, this amounts to a maximal ~1.3-1.4 fold (30-40%) increase in MRK-1 intestinal absorption when MRK-1 was administered to rats in combination with 10-mg/kg ritonavir. Interestingly, at the 10-mg/kg MRK-1 dose, the increase in its AUC upon co-administration with 10-mg/kg ritonavir was 4.3-fold (Table IV); thus, it would appear that all of the increase in AUC of MRK-1 can be accounted for by changes in its systemic $CL_{\text{int}}$ upon co-administration with ritonavir.

We examined also the possibility of quinidine affecting the systemic clearance of MRK-1 via either inhibition of its metabolism and/or biliary, urinary and intestinal secretion or transport. Similar to ritonavir, oral administration of quinidine at 30-mg/kg was found also to impair the systemic clearance of MRK-1, albeit to a lesser extent (~33%). This corresponds to, on average, a ~2 fold reduction in intrinsic hepatic clearance of MRK-1 based on the well-stirred model of liver (Table VI). Thus, quinidine at a 30-mg/kg oral dose, would be expected to increase the AUC of orally co-administered MRK-1 by ~2 fold, based solely on its interaction with the systemic clearance. This indicates that quinidine may maximally increase the extent of MRK-1 absorption (at 2-mg/kg dose) by ~40-50% (Table VI). Interestingly, at the higher MRK-1 dose
(10-mg/kg), there was only a 2-fold increase in the plasma AUC of MRK-1 upon co-administration with quinidine which can all be attributed to the reductions in its systemic clearance (Table V). Thus, it appears that there is little change in the extent of MRK-1 absorption when the compound is administered at a 10-mg/kg dose in combination with quinidine (30-mg/kg). Overall, the data from the rat indicate that the major component of the MRK-1-ritonavir and MRK-1-quinidine interactions arose from inhibition of the systemic clearance of MRK-1. In addition, there may have been a ~30-50% increase in the extent of MRK-1 intestinal absorption when it was administered at a 2-mg/kg dose in combination with either ritonavir (10-mg/kg) or quinidine (10- or 30-mg/kg). Conversely, however, the apparent negligible changes in the intestinal absorption of MRK-1 at the 10-mg/kg dose upon co-administration with ritonavir or quinidine are consistent with nearly complete absorption of the compound at this dose level, likely because of saturation of any P-gp effects (Table III).
Discussion

Combination drug therapy, with intervention at a number of key stages of the viral replication cycle, will likely remain the mainstay of HIV therapy for many years into the future. However, HIV protease inhibitors in general, and ritonavir in particular, have the potential to exhibit significant drug-drug interactions either via the inhibition of CYP isozymes or the efflux transporter P-gp, when given in combination with other compounds. It is clear that management of HIV combination therapy in the clinic requires a thorough understanding of the underlying mechanisms and the biochemical bases of these interactions. Hence, we investigated the role of P-gp and CYP3A in the interaction of an investigational CCR5 receptor antagonist, MRK-1, with ritonavir.

Our in vitro studies demonstrated that MRK-1 was metabolized exclusively by the CYP3A isozymes in liver microsomes from the rat, monkey and the human. Ritonavir proved to be a potent inhibitor of the metabolism of MRK-1 in liver microsomes from all species, with an IC\textsubscript{50} of <0.6 μM. MRK-1 was also a good substrate for the human MDR1 and mouse Mdr1a transporters. The inhibitory effect of ritonavir on P-gp was confirmed by potent inhibition of MRK-1 efflux transport in L-MDR1 cell monolayers with an IC\textsubscript{50} of ~15 μM. Furthermore, the data from the intestinal loop studies in the rat suggested that P-gp inhibitors, verapamil, and especially ritonavir, may profoundly influence the absorption of MRK-1. Incidentally, these data also indicate that MRK-1 is a substrate for efflux transporters at the rat intestinal mucosal surface, and at least in the intestinal loop model, the absorption of MRK-1 was significantly limited by this efflux transport. From these data it appears that ritonavir may exhibit drug-drug
interactions with MRK-1 either via the inhibition of its CYP3A-mediated hepatic metabolism in the liver or P-gp-mediated transport at the intestinal mucosal surface, resulting in reduced systemic clearance and/or its increased absorption, respectively.

Although it is relatively easy to identify the substrates and inhibitors of P-gp using cell lines over expressing this transporter or the isolated intestinal loop preparations, the in vivo significance of these findings is somewhat difficult to ascertain. For example, it is difficult to predict whether the oral absorption of a particular P-gp substrate, as identified from in vitro studies, will be markedly influenced by efflux transport at the intestinal mucosal surface. Similarly, it cannot easily be determined whether a P-gp inhibitor such as ritonavir, which inhibits efflux transport in vitro, would exhibit in vivo drug-drug interactions via this mechanism. The majority of the data in support of the significance of P-gp in determining the disposition and pharmacokinetics of drugs comes from comparative studies in wild type (Mdr1a +/+ ) and Mdr1a deficient (Mdr1a -/- ) mice (Schinkel et al., 1994, 1995, 1996, 1997; van Asperen et al., 1996; Sparreboom et al., 1997; Kim et al., 1998; Iyer et al., 2002). However, there are possible species differences in the substrate specificities of these transporters (Yamazaki et al., 2001). Thus, in vivo studies in other species are needed to truly understand the significance of P-gp transport in determining the disposition of a particular drug candidate during its discovery and development. These studies are, however, difficult to conduct because of a large overlap in the affinities of various substrates and inhibitors of P-gp and CYP3A isozymes so that it becomes difficult to identify whether the observed in vivo interaction is a result of CYP3A or P-gp inhibition or both (Wacher et al., 1995; Kim et al., 1999). Although a few recent reports show that some compounds do indeed exhibit varying degrees of selectivity towards either CYP3A or P-gp (Wandel et al., 1999; Achira et al.,
1999; Dantzig et al., 1999; Cummins et al., 2002), the magnitude of these selectivities is likely not sufficient enough to inhibit one protein without affecting the other in vivo. This is especially true when these compounds are administered orally and high local concentrations are achieved at the intestinal mucosal surface as well as in the portal venous circulation. Hence, with the exception of anticancer drugs that are metabolized by enzymes other than CYP3A, there is little data in literature that directly addresses the role of P-gp in drug disposition in species other than the mouse.

In the present studies, MRK-1 exhibited profound pharmacokinetic interactions following oral co-administration with ritonavir in rats as well as monkeys. From separate studies on MRK-1 disposition in rats and monkeys, we have determined that the systemic clearance of MRK-1 is mediated primarily via hepatic oxidative and conjugative metabolism, with only small contributions from biliary and urinary excretion of the parent compound. Thus, in our studies to investigate the relative significance of P-gp and CYP3A in MRK-1/ritonavir interactions, an approach was chosen where the intestinal and systemic components of these interactions were pharmacokinetically resolved. Inhibition of hepatic CYP3A isozymes likely represents the predominant component of the interaction occurring at the systemic clearance level. Similarly, the increase in MRK-1 absorption via P-gp inhibition is likely a primary factor responsible for the intestinal component of this interaction. Resolution of systemic and intestinal interactions was possible because MRK-1 exhibited a low-to-moderate clearance that was markedly lower than the estimates of hepatic blood flow in both rats and monkeys. This approach assumes that there is negligible intestinal metabolism of the compound; this appeared to be largely true because little metabolism of MRK-1 was detected upon incubation with intestinal microsomes.
from the rat and the monkey. Using this approach, we have demonstrated that the reduction of MRK-1 systemic clearance, probably by way of inhibition of hepatic CYP3A isozymes, on average accounted for ~4.5-fold, out of the observed total 6-fold, increase in the plasma AUC of MRK-1 following oral co-administration of a 2-mg/kg dose with 10-mg/kg ritonavir in rats. The remainder of the increase in plasma MRK-1 AUC (~30-40%) likely arose from its increased absorption via inhibition of P-gp. From the present pharmacokinetic studies, it appeared that the fraction of the administered MRK-1 dose absorbed following oral dosing at 10-mg/kg approached unity. In agreement with this, when a 10-mg/kg dose of MRK-1 was orally administered in combination with 10-mg/kg ritonavir, almost the entire observed increase in MRK-1 plasma AUC could be accounted for by the reductions in systemic clearance and the contribution of the intestinal interaction was minimal.

The role of P-gp in the absorption of MRK-1 in rats was confirmed further by studies in combination with quinidine when an increased and more rapid absorption of MRK-1 was observed. Interestingly, however, a significant component of the quinidine/MRK-1 interactions also appeared to occur via inhibition of systemic clearance of MRK-1. Although the exact mechanism(s) of this interaction remains to be investigated, it could occur via a combination of inhibition of metabolism, and/or biliary and urinary excretion components of MRK-1 clearance. Quinidine, at 10- and 30-mg/kg doses, appeared to result in similar increases in the extent of MRK-1 absorption (40-50%). However, quinidine increased the rate of MRK-1 absorption in a dose-dependent and in a relatively more profound manner (100-300% increase in the first-order absorption rate constant, Table V). These data seem to suggest that P-gp has a greater role in determining the rate rather than the extent of MRK-1 absorption in rats in vivo. This may be
related to the fact that although the net absorption of MRK-1 was slowed down by P-gp mediated efflux transport, the transit time of the drug through the gut was still sufficiently protracted to ensure absorption of the majority of the dose.

In comparison to rats, the nonlinearity in both the bioavailability and estimated oral absorption was somewhat less profound in monkeys and was statistically non-significant. Further, the shape of the plasma concentration vs. time profile of MRK-1 following oral administration suggests a more rapid absorption of the compound in monkeys as compared to rats, i.e., the monkeys appear to exhibit a sharper plasma concentration peak as opposed to a more “flat” profile in rats (Figure 4). A good degree of oral absorption, a less profound nonlinearity in bioavailability with increasing dose and an apparently more rapid absorption profile suggest a limited role of intestinal P-gp in determining the oral absorption of MRK-1 and its interactions with ritonavir in monkeys. The magnitude of interaction between MRK-1 and ritonavir in monkeys as measured by the overall increase in plasma AUC was similar to that in rats. In contrast to rats, however, there was little change in plasma $C_{\text{max}}$ of MRK-1 in monkeys when given in combination with ritonavir. Also, when given in combination with ritonavir, the plasma concentrations of MRK-1 were maintained near $C_{\text{max}}$ for a 6-8 hr period in monkeys which may possibly be related to a more potent and prolonged inhibition of CYP3A-mediated MRK-1 metabolism in this species. It appears that the majority of the increase in AUC of MRK-1 in the above ritonavir co-administration experiments arose from its prolonged half-life or reduced elimination during the 6-8 hr period following dosing and there were minimal, if any, changes in the rate and extent of MRK-1 absorption. Unfortunately, however, the latter could not be confirmed by using
quinidine as a P-gp inhibitor (as in the rat) because of a possible stimulatory effect of this compound on CYP3A isozymes in the monkey (Tang et al., 1999).

Summary and Conclusion

In conclusion, we have demonstrated that ritonavir is a potent inhibitor of the CYP3A-mediated oxidative metabolism of MRK-1 and can also inhibit its P-gp mediated transport. MRK-1 exhibits significant pharmacokinetic interactions upon co-administration with ritonavir, with the plasma AUC increased 4-6 fold in both rats and monkeys. A major mechanism of these interactions is likely the inhibition of hepatic CYP3A-mediated systemic elimination of MRK-1 by ritonavir. At the lower doses of MRK-1 (2-mg/kg), P-gp does appear to play a role in modulating its intestinal absorption and to contribute to its interactions with ritonavir in rats. This is substantiated by the fact that quinidine, a P-gp inhibitor, increases the rate as well as the extent of intestinal absorption of MRK-1 in rats at this dose level. At the higher doses (10-mg/kg), however, the role of P-gp appears to become less significant possibly because of saturation of P-gp mediated transport by high concentrations of the compound at the intestinal mucosal surface. In monkeys, the role of P-gp in determining MRK-1 absorption appears less significant and the increase in MRK-1 absorption by ritonavir via inhibition of intestinal P-gp likely accounts for only a small fraction, if any, of the overall increase in systemic MRK-1 exposure.
Acknowledgments

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References


Legends for Figures

Figure 1. Chemical structure of MRK-1. Symbols * and ** denote the position of [³H] and [¹⁴C] radiolabels in [³H]MRK-1 and [¹⁴C]MRK-1 analogs, respectively.

Figure 2. Effect of ritonavir and cyclosporin A on the efflux transport of MRK-1 in human MDR1 transfected (L-MDR1) cell monolayers. MRK-1 transport was examined in A-to-B direction at a concentration of 10-µM. Cyclosporin A (10-µM) was included as a positive marker for P-glycoprotein inhibition. Each data point is an average of three individual experiments conducted on separate days, each with triplicate determinations.

Figure 3. Effect of P-glycoprotein inhibitors on the absorption of MRK-1 in the isolated rat intestinal loop preparations.

Figure 4. Effect of ritonavir oral co-administration on the pharmacokinetics of MRK-1 in (A) rats and (B) monkeys.

Figure 5. Representative plots of the effect of quinidine oral co-administration on the pharmacokinetics of MRK-1 in rats. (A) MRK-1 (2-mg/kg alone); (B) MRK-1 (2-mg/kg) + quinidine (10-mg/kg); (C) MRK-1 (2-mg/kg) + quinidine (30-mg/kg). Actual plasma concentrations at various times post-dosing (scatter) and the best fit lines from a 1-compartment model with first-order absorption and elimination are shown.

Figure 6. Effect of ritonavir (10-mg/kg) and quinidine (30-mg/kg) oral pre-treatment on the systemic pharmacokinetics of MRK-1 in rats.
Table I. Bi-directional transport of MRK-1 and vinblastine in cell lines transfected with the human MDR1 and mouse Mdr1a transporters and the effect of cyclosporine A (CsA).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transport ratio (B→A/A→B)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRK-1 (10-µM)</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>1.8 ± 0.23</td>
</tr>
<tr>
<td>L-MDR1</td>
<td>10 ± 0.91</td>
</tr>
<tr>
<td>L-Mdr1a</td>
<td>4.3 ± 2.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Each ratio is an average from three individual experiments conducted on separate days, each with a triplicate determination.

All values are mean ± S.D.
Table II. Pharmacokinetic parameters of MRK-1 following *i.v.* and oral administration in male Sprague-Dawley rats (n=3 per group) and rhesus monkeys (n=4 per group).

<table>
<thead>
<tr>
<th></th>
<th>IV Dose</th>
<th>Oral Dose</th>
<th>Oral Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5-mg/kg</td>
<td>2-mg/kg</td>
<td>2-mg/kg</td>
</tr>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0–∞&lt;/sub&gt; (ng.hr/mL)</td>
<td>699 ± 147</td>
<td>3,462 ± 1,318</td>
<td>978 ± 727</td>
</tr>
<tr>
<td>CL&lt;sub&gt;p&lt;/sub&gt; (mL/min/kg)</td>
<td>12.3 ± 2.9</td>
<td>10.9 ± 5.3</td>
<td>-</td>
</tr>
<tr>
<td>CL&lt;sub&gt;blood&lt;/sub&gt; (mL/min/kg)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.8 ± 4.7</td>
<td>17.6 ± 8.5</td>
<td>-</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>1.5 ± 0.7</td>
<td>2.9 ± 1.2</td>
<td>-</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>F (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td><strong>Rhesus Monkeys</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0–∞&lt;/sub&gt; (ng.hr/mL)</td>
<td>691 ± 171</td>
<td>3,322 ± 284</td>
<td>884 ± 312</td>
</tr>
<tr>
<td>CL&lt;sub&gt;p&lt;/sub&gt; (mL/min/kg)</td>
<td>12.1 ± 2.5</td>
<td>10.1 ± 0.9</td>
<td>-</td>
</tr>
<tr>
<td>CL&lt;sub&gt;blood&lt;/sub&gt; (mL/min/kg)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.6 ± 4.0</td>
<td>16.3 ± 1.4</td>
<td>-</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>1.8 ± 0.6</td>
<td>1.2 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>2.1 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>2.4 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>-</td>
</tr>
</tbody>
</table>
Bioavailability in rats was determined using average AUC data from i.v. bolus and oral dosing studies in separate groups of animals.

Bioavailability in monkeys was determined using AUC data from i.v. bolus and oral administration experiments conducted with a randomized two-way cross over design in each monkey.

Calculated by dividing plasma clearance in each animal with the average in vitro blood-to-plasma ratio.

All values are mean ± S.D.
Table III. Estimates of fraction of MRK-1 oral dose absorbed in rats and adult rhesus monkeys.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: MRK-1 (0.5-mg/kg IV)</td>
<td>$CL_{int(blood)}^A$ (mL/min/kg)</td>
<td>28.6</td>
</tr>
<tr>
<td>B: MRK-1 (2-mg/kg PO)</td>
<td>$CL_{int(blood)}/F_a^B$ (mL/min/kg)</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>Fraction Absorbed ($F_a$) = $CL_{int(blood)}^A/[CL_{int(blood)}^B/F_a^B]$</td>
<td>0.52</td>
</tr>
<tr>
<td>C: MRK-1 (2-mg/kg IV)</td>
<td>$CL_{int(blood)}^C$ (mL/min/kg)</td>
<td>24.1</td>
</tr>
<tr>
<td>D: MRK-1 (10-mg/kg PO)</td>
<td>$CL_{int(blood)}/F_a^D$ (mL/min/kg)</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>Fraction Absorbed ($F_a$) = $CL_{int(blood)}^C/[CL_{int(blood)}^D/F_a^D]$</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Adult Rhesus Monkeys</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: MRK-1 (0.5-mg/kg IV)</td>
<td>$CL_{int(blood)}^E$ (mL/min/kg)</td>
<td>34.6</td>
</tr>
<tr>
<td>F: MRK-1 (2-mg/kg PO)</td>
<td>$CL_{int(blood)}/F_a^F$ (mL/min/kg)</td>
<td>60.8</td>
</tr>
<tr>
<td></td>
<td>Fraction Absorbed ($F_a$) = $CL_{int(blood)}^E/[CL_{int(blood)}^F/F_a^F]$</td>
<td>0.57</td>
</tr>
<tr>
<td>G: MRK-1 (2-mg/kg IV)</td>
<td>$CL_{int(blood)}^G$ (mL/min/kg)</td>
<td>25.5</td>
</tr>
<tr>
<td>H: MRK-1 (10-mg/kg PO)</td>
<td>$CL_{int(blood)}/F_a^H$ (mL/min/kg)</td>
<td>32.3</td>
</tr>
</tbody>
</table>
Fraction Absorbed ($F_a$) = 0.79

$$CL^{\text{G}}_{\text{int(blood)}}/[CL^{\text{H}}_{\text{int(blood)}}/F_a]$$

Calculation of intrinsic clearances and fraction absorbed was performed according to the well-stirred model of organ clearance. Details are described under Methods in the Pharmacokinetic Analyses section.
Table IV. Effect of 10-mg/kg ritonavir oral co-administration on the pharmacokinetics of MRK-1 in rats (n=4 per group) and monkeys (n=3 and 4 per group for 2 and 10-mg/kg MRK-1, respectively).

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>MRK-1 Dose (2-mg/kg PO)</th>
<th>MRK-1 Dose (10-mg/kg PO)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + Ritonavir</td>
<td>Control + Ritonavir</td>
</tr>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng.hr/mL)</td>
<td>978±727</td>
<td>6,198±1,314</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>321±214</td>
<td>1,561±364</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>0.4±0.1</td>
<td>2.0±1.4</td>
</tr>
<tr>
<td><strong>Adult Monkeys</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng.hr/mL)</td>
<td>936±360</td>
<td>5,650±1,873</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>569±323</td>
<td>622±175</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>0.4±0.1</td>
<td>3.3±1.2</td>
</tr>
</tbody>
</table>

All values are mean ± S.D.
Table V. Effect of oral co-administration of quinidine on the pharmacokinetics of MRK-1 in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dosing Group</th>
<th>2-mg/kg MRK-1</th>
<th>10-mg/kg MRK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRK-1 Alone</td>
<td>MRK-1 + 10-mg/kg Quinidine</td>
<td>MRK-1 + 30-mg/kg Quinidine</td>
</tr>
<tr>
<td>AUC₀-∞ (ng.hr/mL)</td>
<td>640 ± 444</td>
<td>1,744 ± 619</td>
<td>1,861 ± 344</td>
</tr>
<tr>
<td>C_max (ng/mL)</td>
<td>144 ± 106</td>
<td>530 ± 133</td>
<td>714 ± 161</td>
</tr>
<tr>
<td>K_{abs} (hr⁻¹)</td>
<td>2.3 ± 1.0</td>
<td>5.2 ± 2.5</td>
<td>11 ± 4.4</td>
</tr>
<tr>
<td>K_{el} (hr⁻¹)</td>
<td>0.37 ± 0.07</td>
<td>0.43 ± 0.06</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>T_{max} (hr)</td>
<td>0.94 ± 0.21</td>
<td>0.52 ± 0.16</td>
<td>0.32 ± 0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10-mg/kg MRK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₀-∞ (ng.hr/mL)</td>
</tr>
<tr>
<td>C_max (ng/mL)</td>
</tr>
<tr>
<td>K_{abs} (hr⁻¹)</td>
</tr>
<tr>
<td>K_{el} (hr⁻¹)</td>
</tr>
<tr>
<td>T_{max} (hr)</td>
</tr>
</tbody>
</table>

a. K_{abs}, K_{el} and T_{max} were obtained by fitting of the data to a one-compartment model with first-order absorption and elimination.
b. Not determined.

All values are mean ± S.D.
Table VI. Effect of oral ritonavir and quinidine administration on the systemic pharmacokinetics of MRK-1 in rats (n=3 per group). In all dosing groups, MRK-1 was administered at a dose of 0.5-mg/kg via an i.v. bolus injection.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MRK-1 Dosing Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{0-\infty} (ng.hr/mL)</td>
<td>500 ± 150</td>
</tr>
<tr>
<td>(CL_p) (mL/min/kg)</td>
<td>17.6 ± 4.9</td>
</tr>
<tr>
<td>(CL_{\text{blood}}) (mL/min/kg)(^a)</td>
<td>28.5 ± 7.9</td>
</tr>
<tr>
<td>(CL_{\text{int}}) (mL/min/kg)(^b)</td>
<td>54.3 ± 25.6</td>
</tr>
<tr>
<td>(V_{\text{ss}}) (L/kg)</td>
<td>0.78 ± 0.14</td>
</tr>
<tr>
<td>(T_{1/2}) (hr)</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>0.75 ± 0.09</td>
</tr>
</tbody>
</table>

\(^a\) calculated by dividing plasma clearance in each animal with the average \textit{in vitro} blood-to-plasma ratio.  
\(^b\) calculated using the \(CL_{\text{blood}}\) values and an assumed rat hepatic blood flow of 65 mL/min/kg. A well-stirred model of hepatic clearance was assumed for this calculation.

All values are mean ± S.D.
Figure 1
Figure 2

Inhibitor Concentration (µM) 0 2 0 4 0 6 0 8 0

P-Glycoprotein Mediated MRK-1 Transport (% of Control)

Ritonavir IC₅₀ = 15.7 ± 9.0 µM (n=3)

Ritonavir
Cyclosporin A (10-µM)

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Figure 3

![Graph showing MRK-1 Absorbed (ng) over time (min) for different conditions: 0.1 mg MRK-1, 0.1 mg MRK-1 + 0.1 mg Verapamil, and 0.1 mg MRK-1 + 0.1 mg Ritonavir.](chart.png)
Figure 4

A

- MRK-1 (2 mg/kg) Alone
- MRK-1 (2 mg/kg) + Ritonavir (10 mg/kg)
- MRK-1 (10 mg/kg) Alone
- MRK-1 (10 mg/kg) + Ritonavir (10 mg/kg)

B

- MRK-1 alone (2-mg/kg)
- MRK-1 (2-mg/kg) + Ritonavir (10-mg/kg)
- MRK-1 alone (10-mg/kg)
- MRK-1 (10-mg/kg) + Ritonavir (10-mg/kg)
Figure 5

A B C
Figure 6

- 0.5 mg/kg IV MRK-1
- 0.5 mg/kg IV MRK-1, 0.5 h after 10 mg/kg Ritonavir PO
- 0.5 mg/kg IV MRK-1, 0.5 h after 30 mg/kg Quinidine PO

MRK-1 Plasma Concentration (ng/mL) vs Time (h)