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Sirolimus Oral Absorption in Rats Is Increased by Ketoconazole but Is Not Affected by D-α-Tocopheryl Poly(Ethylene Glycol 1000) Succinate

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ABSTRACT

The contributions of cytochrome P450 3A (CYP3A) and P-glycoprotein to sirolimus oral bioavailability in rats were evaluated by coadministration of sirolimus (Rapamune) with the CYP3A inhibitor ketoconazole or the P-glycoprotein inhibitor D-α-tocopheryl poly(ethylene glycol 1000) succinate (TPGS). Groups of six male Sprague-Dawley rats (250–300 g) were administered Rapamune (1 mg/kg) by oral gavage, alone and with ketoconazole (30 mg/kg) or TPGS (50 mg/kg). Sirolimus levels were measured in whole blood over a 6-h time course. Sirolimus C_{max} (6.6 $\pm$ 1.6 versus 26 $\pm$ 7 ng/ml) and area under the concentration versus time curve from 0 to 6 h (AUC_{0-6}) (22 $\pm$ 7 versus 105 $\pm$ 27 ng $\cdot$ h/ml) were increased 3- to 5-fold by ketoconazole. Median T_{max} (1.5–2 h) was unchanged. TPGS had no effect on sirolimus absorption. The interaction of sirolimus with P-glycoprotein was also evaluated in vitro using HCT-8 and Caco-2 cell monolayers. Consistent with published reports, sirolimus was a good inhibitor of P-glycoprotein, inhibiting polarized basolateral-to-apical flux of rhodamine 123 with an IC_{50} of 0.625 to 1.25 μM (cyclosporine caused >80% inhibition at 5 μM). Sirolimus did not demonstrate significant polarized flux in either direction using the same monolayers (basolateral-to-apical flux was <2 times the apical-to-basolateral). Moreover, sirolimus flux was not impacted by cyclosporine, suggesting that it does not undergo P-glycoprotein-mediated transport in this system. The lack of significant sirolimus transport by P-glycoprotein may, in part, explain the lack of a TPGS effect on sirolimus absorption in rats.

Sirolimus (Rapamune) is a macrocyclic lactone used for immunosuppression following renal transplantation (Rapamune (sirolimus) Oral Solution. Approved Product Labeling). Sirolimus suffers from poor oral bioavailability due, in large part, to extensive presystemic metabolism by cytochrome P450 3A (CYP3A). Clinical drug interactions have demonstrated that sirolimus levels are significantly increased when administered with cyclosporine, an established CYP3A substrate (Kaplan et al., 1998). A more recent study in healthy volunteers found that coadministration of the potent CYP3A inhibitor ketoconazole increased sirolimus oral bioavailability up to 11-fold in healthy volunteers, primarily through inhibition of sirolimus metabolism in the small intestine (Benet, 2000). Many CYP3A substrates are also transported by the drug efflux pump P-glycoprotein (P-gp) (Wacher et al., 1998). Sirolimus is an established P-gp inhibitor (Arceci et al., 1992); however, P-gp-mediated transport of sirolimus has not been definitively demonstrated. One report found 18-fold greater basolateral-to-apical (efflux) versus apical-to-basolateral (absorptive) transport across Caco-2 cell monolayers; however, this efflux was only partially blocked by the P-gp inhibitor verapamil, and a role for the multidrug resistance-related proteins (MRPs) was also proposed (Crowe and Lemaire, 1998). A subsequent study found the exact opposite, reporting highly polarized transport from the apical to basolateral compartments of Caco-2, HCT-8, and T84 monolayers (Dias and Yatscoff, 1994, 1996). Sirolimus metabolites were found to sort almost exclusively to the mucosal side of pig intestinal tissue in an Ussing chamber; however, polarized flux of sirolimus itself was not evaluated in these studies (Lampen et al., 1998).

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ABBREVIATIONS: CYP3A, cytochrome P450 3A; P-gp, P-glycoprotein; MRP, multidrug resistance-related protein; TPGS, D-α-tocopheryl poly(ethylene glycol 1000) succinate; HPLC-MS, high-pressure liquid chromatography-mass spectroscopy; QC, quality control; AUC, area under the concentration versus time curve; P450, cytochrome P450; R123, rhodamine 123; M1–3, unidentified sirolimus metabolites; TBS, Tween-phosphate-buffered saline; 39-ODM, 39-*O*-desmethylsirolimus; CDNB, 1-chloro-2,4-dinitrobenzene.

The current work evaluates the interaction of sirolimus with P-gp in vitro by measuring sirolimus flux across Caco-2 and HCT-8 cell monolayers and by determining the impact of sirolimus on the polarized transport of rhodamine 123 (R123), an established P-gp substrate. The contribution of CYP3A to sirolimus oral bioavailability was confirmed by measuring the effect of ketoconazole (a CYP3A inhibitor) on sirolimus oral bioavailability in rats. The effect of ketoconazole on sirolimus pharmacokinetics was compared with that of the solubility enhancer and P-gp inhibitor (Dintaman and Silverman, 1999; Yu et al., 1999) D-α-tocopheryl poly(ethylene glycol 1000) succinate (TPGS). TPGS has previously been shown to increase cyclosporine oral absorption 2- to 3-fold in male rats (Wacher et al., 2002). No published data are available describing a sirolimus-ketoconazole interaction in rats; however, ketoconazole inhibits sirolimus metabolism in rat intestinal and hepatic microsomes (Lampen et al., 1998). Moreover, coadministration of the CYP3A and P-gp inhibitor cyclosporine resulted in 2- to 11-fold increases in sirolimus oral bioavailability and caused dose-dependent increases in sirolimus tissue concentrations in this species (Stepkowski et al., 1996; Napoli et al., 1998).

Materials and Methods

Materials. Rapamune (sirolimus) Oral Solution (1 mg/ml) (Wyeth Laboratories, Philadelphia, PA) was commercially available. Unformulated sirolimus was obtained from AG Scientific (San Diego, CA). Unformulated cyclosporine was obtained from Sigma-Aldrich (St. Louis, MO). TPGS was obtained from Eastman Kodak Co. (Rochester, NY). Ketoconazole was obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA).

Animals. Male Sprague-Dawley rats (250–300 g body weight) with cannulae inserted into the jugular vein were purchased from Harlan (Madison, WI). Catheter patency was maintained using a heparin lock. Dosing and blood sampling were conducted by Northview Pacific Laboratories Inc. (Hercules, CA). Protocols and standard operating procedures were reviewed by the site's Internal Animal Care and Use Committee. Animal handling was conducted according to guidelines established by the Animal Welfare Act. Animals were individually housed at 18–26°C and allowed free movement and access to water. Rats were fed standard laboratory rodent diet during a minimum 1-day acclimatization period but were fasted from 12 h before dose administration and were not administered food throughout the study.

Doses and Administration. Groups of six rats were administered sirolimus (1 mg/kg as the Rapamune formulation) by oral gavage, alone and with ketoconazole (30 mg/kg, study 1; 10 mg/kg, study 2) or TPGS (50 mg/kg). Rapamune (2.5 ml) was mixed with a suspension of ketoconazole (25 or 75 mg) in ethanol (0.25 ml), and the dose was diluted with 0.9% saline to a final volume of 25 ml. Alternatively, Rapamune (1.0 ml) was mixed with TPGS (50 mg) in ethanol (0.1 ml), and the doses were diluted with 0.9% saline to a final volume of 10 ml. The reference dose was Rapamune diluted in saline. All doses formed milky white emulsions on saline dilution. Rats were administered 10 ml/kg of each emulsion using a standard gavage needle.

Initial dilution of Rapamune concentrates in saline resulted in milky white emulsions regardless of the dosage form. The saline-diluted control, and ketoconazole- and TPGS-containing Rapamune emulsions were stable for at least 3 days, with no evidence of settling or precipitation. A relatively high gavage volume (10 ml/kg) of each dose was administered; however, no reflux or dose spillage from the rats was observed. More concentrated sirolimus gavage emulsions (and hence lower gavage volumes) were precluded by increased viscosity and difficulty in handling and dosing.

Blood Sampling and Analysis. Serial blood samples (500 μl) were drawn prior to the dose (time 0) and at 0.5, 1, 1.5, 2, 3, 4, and 6 h postdose through a cannula inserted into the jugular vein. Blood volume was replaced with saline after each sample. Whole blood samples were collected in Microtainer tubes (BD Biosciences, Franklin Lakes, NJ) containing sodium EDTA anticoagulant and were stored in the refrigerator prior to extraction and analysis. No hemolysis or coagulation was observed for blood samples over the study period.

Sirolimus blood extraction used modifications of published methods (Streit et al., 1996b; Maleki et al., 2000). Whole blood samples (400 μl) were extracted by vortex mixing for 60 s with 400 μl of extraction solvent (70% methanol/30% 200 mM zinc sulfate) and 20 μl of internal standard solution (100 nM cyclosporine in acetonitrile). Precipitated materials were separated by centrifugation (3000 rpm for 10 min), and then the supernatants were extracted with *n*-butyl chloride (2 ml). Phases were separated by centrifugation; then, the organic phase was removed and evaporated to dryness under nitrogen. Residues were reconstituted in 150 μl of solvent (70:9:21 methanol/acetonitrile/pH 3 water), and 50 μl was analyzed by HPLC-MS.

HPLC-MS analysis utilized a Hewlett Packard Series 1100 chromatography system with detection using a Series 1100 mass-selective detector. Sirolimus was analyzed on a narrow-pore Rainin Microsorb C-18 column (4.6 × 150 mm; 2 μm) maintained at 50°C. UV detection was at 266 nm. Solvent flow rate was 0.5 ml/min. Elution of sirolimus from the column utilized a binary solvent gradient system in which solvent A was 100 mM sodium formate (pH 3) and solvent B was acetonitrile. The column was initially equilibrated at 50% solvent A and 50% solvent B. Immediately upon sample injection, the concentration of B was increased linearly over 10 min to a final concentration of 100%. The system was returned to the original conditions and equilibrated for 3 min before injecting another sample. Retention times for sirolimus and cyclosporine internal standard were 7.3 min and 8.7 min, respectively. Sodium adducts of sirolimus ($M - Na^+$, $m/z = 936.6$) and cyclosporine ($M - Na^+$, $m/z = 1224.7$) were analyzed by electrospray ionization-mass spectrometry using selective ion monitoring. The mass spectrometer was run in the positive ion mode with N_2 drying gas flow of 12 l/min, drying gas temperature 350°C, nebulizer pressure 50 psi gauge, chamber current 0.59 μA, capillary current 31 nA, and capillary voltage 4000 V.

Sirolimus blood concentrations were quantified by comparison with standard curves generated from spiked blood samples extracted in the same manner as the test samples. Two rats from each group were analyzed each day together with duplicate standard samples and triplicate quality control (QC) samples. Standard curve samples (2–50 ng/ml) were prepared fresh each analysis day. QC samples (5, 20, and 50 ng/ml) were prepared on day 1 and maintained in the refrigerator with the other test samples. Standard curves were linear over the range tested with r^2 values >0.99. Mean ± S.D. (CV%) concentrations in 5, 20, and 50 ng/ml QC samples were 4.9 ± 0.3 (6.5), 20.5 ± 0.7 (3.3), and 46.9 ± 1.8 (3.8) ng/ml. Observed sirolimus concentrations were 98 ± 6%, 103 ± 3%, and 94 ± 4% of the respective nominal concentrations in QC samples, which are well within acceptable validation criteria. The lower limit of quantitation was 2 ng/ml.

Pharmacokinetic and Statistical Analysis. Peak blood sirolimus concentrations (C_{max}) and time to achieve these concentrations (T_{max}) were measured directly from concentration versus time profiles. Area under the concentration versus time curve from 0 to 6 h (AUC_{0-6}) was calculated using the linear trapezoidal method. For studies with three or more doses, data were compared using one-way analysis of variance, or analysis of variance based on ranks, with the Dunnett post hoc comparison. For studies with only two doses, data were compared using an unpaired *t* test (normally distributed data) or the Mann-Whitney rank-sum test (SigmaStat version 2.0; SPSS Science, Chicago, IL).

Sirolimus Metabolism. Sirolimus (10 μM) and inhibitor or inhibitor vehicle were preincubated with liver microsomes from a human donor (100 μg/ml) or dexamethasone-induced rats (100 μg/ml) (prepared as in Wacher et al., 2002) and diethylenetriamine pentaacetic acid (1 mM) in 100 mM phosphate buffer, pH 7.4, for 5 min at 37°C.

Metabolic reactions were started by addition of NADPH to give a final concentration of 1 mM and a final volume of 0.5 ml. Reactions were stopped after 10 min by addition of 200 μl of stop solution (94:6 acetonitrile/glacial acetic acid). Protein was precipitated by centrifugation (3000 rpm for 10 min); then, supernatants were analyzed for sirolimus and its oxidation products by HPLC with UV detection. Identical experiments were conducted using Supersomes (BD Gentest, Woburn, MA) containing CYP3A4 + cytochrome b_5 + P450 reductase (50 pmol of CYP3A/ml), CYP3A4 + P450 reductase (100 pmol of CYP3A/ml), and CYP3A5 + P450 reductase (100 pmol of CYP3A/ml). All experiments were conducted in triplicate and compared to reactions with inhibitor and substrate but without NADPH. Possible interfering peaks in the HPLC traces were identified by analysis of metabolic incubations with and without NADPH in the absence of substrate.

Sirolimus and metabolites were separated on a Rainin Microsorb-MV C-8 column (5 μm; 4.6 mm $\times$ 250 mm) using a binary solvent gradient system. Solvent A was water acidified to pH 3 with phosphoric acid, and solvent B was acetonitrile. Solvent flow rate was 1.0 ml/min and column temperature was 50°C. The initial mobile phase consisted of 50% solvent A and 50% solvent B. Immediately upon sample injection, the concentration of B was increased linearly to 75% over 10 min (2.5% per minute). At 15 min, the system was returned to the initial conditions (50% B) and equilibrated for 2 min before the next run. Sirolimus and metabolites were analyzed by UV detection at 276 nm.

Microsomal metabolism screens were further validated by measuring the effect of an anti-CYP3A4 monoclonal antibody (BD Gentest) on sirolimus metabolism. The desired volumes of antibody solution were diluted to 10 μl with Tris buffer. Sirolimus (10 μM) and diluted antibody (10 μl) were preincubated with human liver microsomes (100 μg/ml) for 15 min on ice according to the vendor's specifications. Incubation mixtures were transferred to a 37°C water bath, and metabolic reactions were started by addition of NADPH to give a final concentration of 1 mM and a final volume of 0.5 ml. Reactions were stopped after 30 min, extracted, and analyzed as described above.

Cell Monolayers. HCT-8 cells, derived from a human ileocecal adenocarcinoma, were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% horse serum, 1 mM sodium pyruvate, and 0.01 mg/ml gentamicin. Caco-2 cells (American Type Culture Collection) were grown in Eagle's minimum essential medium with nonessential amino acids, 10% fetal bovine serum, and 50 μg/ml gentamicin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. For transport studies, HCT-8 cells were plated at a density of 50×10^5 cells/cm^2 on 24-mm-diameter, 0.4 μm pore size Transwell polyester membranes (Corning Glassworks, Corning, NY). Caco-2 cells were plated at a density of 75×10^5 cells/cm^2 on 24-mm-diameter, 0.4 μm pore size collagen-coated Transwell polyester membranes (Corning). Culture medium was replaced every 2 days until a tight cell monolayer was formed as measured by transepithelial electrical resistance and preliminary R123 permeability measurements. HCT-8 cells were used approximately 5 days after plating, whereas Caco-2 cells were used 14 to 21 days after plating.

R123 Flux. R123 was added at a final concentration of 15 μM to either the basolateral or the apical compartments of the HCT-8 or Caco-2 cell monolayers. Media aliquots (200 μl) were taken at 2, 4, and 6 h from the opposite chamber, and the fluorescence of R123 was measured at excitation wavelength 485 nm and emission wavelength 530 nm (Chaudhary et al., 1992; Egudina et al., 1993). For P-gp inhibition experiments, sirolimus was added as an inhibitor to both compartments. All experiments were performed in triplicate.

Sirolimus Flux. P-gp-mediated transport of sirolimus was examined across both HCT-8 and Caco-2 cell monolayers. Sirolimus (0.1 μM) was added to either the basolateral or the apical side, and 200-μl aliquots were taken at 2, 4, and 6 h from the opposite chamber. The transport medium was similar to the maintenance medium but did not contain serum. Samples were extracted by addition of 200 μl of stop solution (94:6 acetonitrile/acetic acid) followed by 10 μl of internal standard (1 μM cyclosporine) and 500 μl of methyl-*tert*-butyl ether. After vortex mixing, the phases were separated by centrifugation (3000 rpm for 10 min); then, the methyl-*tert*-butyl ether phase was transferred and evaporated to dryness under nitrogen. Samples were reconstituted in 200 μl of injection solvent (75:25 acetonitrile/sulfuric acid, pH 3); then, 50 μl was injected for liquid chromatography-MS using a modification of the method described above. Analytes were separated on a Beckman reverse phase C-18 cartridge (5 μm; 4.6 mm $\times$ 45 mm) using a binary solvent gradient system. Solvent A was 1 mM sodium formate (pH 3) and solvent B was 80:20 methanol/acetonitrile. Solvent flow rate was 0.5 ml/min, and column temperature was 35°C. The initial mobile phase consisted of 20% solvent A and 80% solvent B. Immediately upon sample injection, the concentration of B was increased to 90% over 10 min (1% per minute) which was maintained for 3 min. The system was returned to the original conditions and equilibrated for 3 min before injecting another sample. Drug concentrations were quantified by comparison to standard curves. Standard curves (duplicate) were linear over the range tested (1–100 nM; $r^2 \geq 0.99$). The lower limit of quantitation was 1 nM.

Western Blot Analysis. Cell membranes were isolated using standard centrifugation techniques. Cell pellets were resuspended in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl, 1 mM $MgCl_2$, and a protease inhibitor cocktail (pepstatin, leupeptin, Pefabloc) and then homogenized with a Dounce homogenizer. Homogenates were centrifuged (400*g* for 5 min, 4°C). The resulting supernatants then underwent ultracentrifugation (100,000*g* for 30 min, 4°C). The cell membrane pellet was resuspended in lysis buffer and stored at −80°C prior to gel electrophoresis. Protein concentration was determined using the method of Bradford (1976).

P-gp expression was measured using the MDR-specific antibody C219 (Chemicon, Temecula, CA). Ten (LLC-PK1 cells) or 20 μg (HCT-8, Caco-2 cells) of protein was resolved in an 8% SDS-polyacrylamide gel at 120 V. The samples were transferred to polyvinylidene difluoride membranes (Novex, San Diego, CA) at 100 mA overnight. The blots were stained with Ponceau S-solution [0.1% Ponceau S (w/v) in 5% acetic acid (v/v)] to confirm equal protein loading in all lanes. The blots were blocked with 5% nonfat milk made in 0.1% Tween-phosphate-buffered saline (TBS), rinsed briefly in TBS, and then incubated overnight with C219 antibody in 0.1% nonfat milk/TBS. Finally, the blots were incubated for 1 h with a horseradish peroxidase-labeled antibody (Amersham Biosciences Inc., Piscataway, NJ) at a 1:2000 dilution in 0.1% nonfat milk/TBS, and developed using the Pierce Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL).

Results

Sirolimus Pharmacokinetics. Pharmacokinetic data for studies with sirolimus are presented in Table 1, and concentration versus time profiles for sirolimus are presented in Fig. 1. Sirolimus absorption in all groups was highly variable. Both studies utilized identical dose preparation and administration procedures. Coadministration of 30 mg/kg ketoconazole caused 3- to 5-fold increases in sirolimus C_{max} and AUC_{0-6} while reducing T_{max} by half an hour. Reducing the ketoconazole dose to 10 mg/kg caused 5- to 6-fold increases in sirolimus C_{max} and AUC_{0-6} without affecting T_{max}. This effect was not statistically different from that observed with the 30 mg/kg ketoconazole dose. TPGS (50 mg/kg) had no effect on sirolimus C_{max} AUC_{0-6} or T_{max}.

Sirolimus Metabolism. Sirolimus microsomal incubations utilized a saturating substrate concentration with microsomal protein concentration and incubation time optimized for linearity of metabolite formation. Several NADPH-dependent metabolite peaks were observed in the HPLC traces from human liver microsomal incubations, represented by a triplet at 8.1 to 8.7 min (M1–3) and a single peak at 10.5 min. This metabolite profile is similar to that ob-

TABLE 1
Effect of ketoconazole and TPGS on the oral absorption of sirolimus (1 mg/kg) in rats
Data are mean ± S.D. (CV%) except T_{max} values, which are median (range).

Parameter	Additive (mg/kg)	Study 1 (n = 5)	Study 2 (n = 3)
C_{max} (ng/ml)	Control	6.6 ± 1.6 (25)	7.4 ± 4.3 (58)
	Ketoconazole (10)		42 ± 20 (48)*
	Ketoconazole (30)	26 ± 7 (27)*	
	TPGS (50)	5.7 ± 1.9 (34)	
T_{max} (h)	Control	2.0 (2–2)	1.5 (1.5–3)
	Ketoconazole (10)		2.0 (1.5–2)
	Ketoconazole (30)	1.5 (1.5–1.5)*	
	TPGS (50)	1.5 (1–1.5)*	
AUC_{0-6} (ng · h/ml)	Control	22 ± 7 (32)	25 ± 23 (92)
	Ketoconazole (10)		141 ± 60 (43)*
	Ketoconazole (30)	105 ± 27 (26)*	
	TPGS (50)	17 ± 9 (53)	

* Statistically different from control, $p < 0.05$.

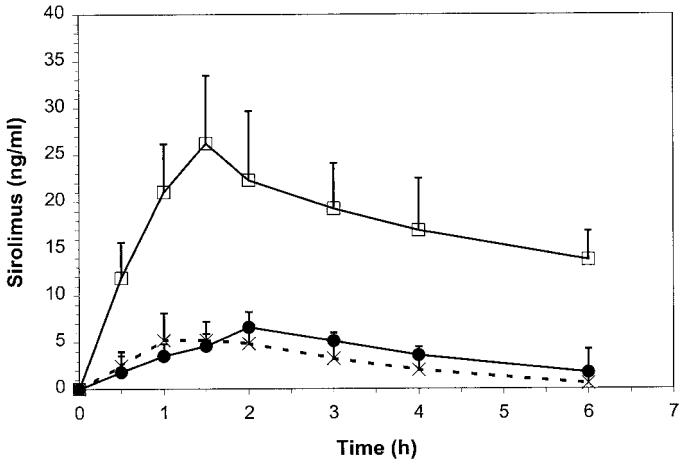

Fig. 1. Mean sirolimus concentration versus time profiles for five rats administered Rapamune alone (●), with TPGS (×), or with ketoconazole (□).

served in published studies with human liver microsomes (Streit et al., 1996a). The metabolite profile obtained with dexamethasone-induced rat liver microsomes was identical to that obtained for human liver microsomes. Metabolite levels in microsomal incubations were similar between the species when normalized for microsomal protein content; however, metabolic activity was 50% lower in the induced rat liver microsomes compared with the human liver microsomes when normalized for total P450 content. HPLC-MS analysis showed that the molecular weight of the metabolite at 10.5 min ($M - Na^+$ m/z = 922.5) was 14 units lower than that of sirolimus ($M - Na^+$ m/z = 936.6), consistent with a demethylated product. The molecular weight of the major peak in the triplet group ($M - Na^+$ m/z = 952.5) was 16 units higher than that of sirolimus, suggesting a hydroxylated metabolite. Based on comparison of the relative retention times and metabolite levels to published reports, the single demethylated peak was identified as 39-*O*-demethylsirolimus (39-ODM). Sirolimus metabolism in human liver microsomes was inhibited in a dose-dependent manner by an anti-CYP3A4 monoclonal antibody. M1–3 levels were decreased by 10, 13, 34, 49, and 57% in the presence of 0.5, 1, 2, 5, and 10 μl of antibody, respectively. 39-ODM levels were decreased by 10, 15, 44, 62, and 73% at these antibody concentrations. Coincubation with ketoconazole (1 μM) reduced levels of M1–3 and 39-ODM by 71% and 78%, respectively, in human liver microsomes, whereas >80% inhibition was observed in dexamethasone-induced rat liver microsomes.

The metabolite profile obtained from incubations with CYP3A4 Supersomes was identical to that seen in human liver microsomes. As observed for other CYP3A substrates (Guengerich, 1999; Wacher et al., 2002), addition of cytochrome b_5 significantly increased the extent of CYP3A4-mediated metabolism in this system, doubling levels of both M1–3 and 39-ODM. M1–3 levels measured in incubations with CYP3A5 were approximately 80% of those observed for CYP3A4 in the absence of cytochrome b_5. In contrast, 39-ODM levels in incubations using CYP3A5 were only 20% of the levels obtained with CYP3A4. Ketoconazole (1 μM) reduced levels of M1–3 and 39-ODM by 68% and 78%, respectively in incubations with CYP3A4. Similar reductions in the levels of M1–3 (64%) and 39-ODM (69%) were effected by ketoconazole in incubations with CYP3A4 + cytochrome b_5. Ketoconazole was less effective as an inhibitor of sirolimus metabolism by CYP3A5, reducing levels of M1–3 by only 38% and 39-ODM by 54%.

P-Glycoprotein Expression. Expression of P-gp was examined in the HCT-8 and Caco-2 intestinal cell lines by Western blot analysis using the MDR-reactive antibody C219 (Fig. 2). Membrane proteins isolated from LLC-PK1 cells transfected with the human MDR1 cDNA are also shown as a positive control. P-gp was expressed in both HCT-8 and Caco-2 cells although at higher levels in the HCT-8 cells.

R123 Flux. Sirolimus was tested for its capacity to inhibit the polarized transport of R123, a well established P-gp substrate (Chaudhary et al., 1992; Egudina et al., 1993), across HCT-8 cell monolayers and Caco-2 cell monolayers (Fig. 3). R123 was actively transported by P-gp in the basolateral-to-apical direction across epithelial cell monolayers. In the absence of sirolimus there was 6.7-fold greater R123 flux in the basolateral-to-apical (excretory) versus the apical-to-basolateral (absorptive) direction across HCT-8 cell monolayers. Addition of sirolimus resulted in a dose-dependent decrease in

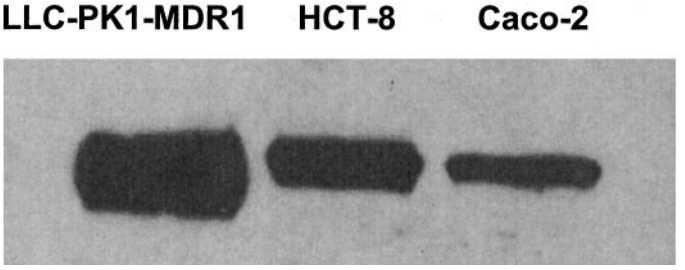

Fig. 2. Western immunoblot analysis of P-gp expression in HCT-8 and Caco-2 cells as well as MDR1-transfected LLC-PK1 cells.

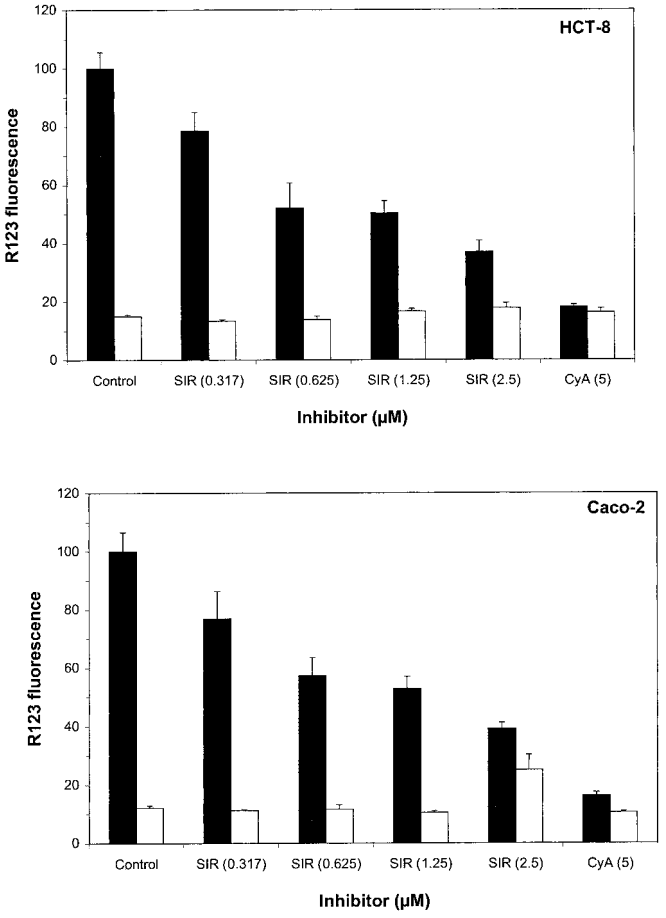

Fig. 3. Inhibition of R123 flux across HCT-8 and Caco-2 cell monolayers by sirolimus (SIR) and cyclosporine (CyA). Dark bars represent flux in the basolateral-to-apical direction. Light bars represent flux in the apical-to-basolateral direction.

R123 basolateral-to-apical transport, with an IC_{50} of approximately 1.25 μM. Sirolimus also inhibited P-gp-mediated transport of R123 in the Caco-2 cells. At 6 h, the basolateral-to-apical efflux of R123 was 8.2 times greater than the apical-to-basolateral influx. Addition of sirolimus resulted in a dose-dependent inhibition of R123 flux with an IC_{50} between 0.625 and 1.5 μM in these cells. Cyclosporine, an established inhibitor of P-gp-mediated transport, diminished the basolateral-to-apical transport of R123 by approximately 85% when used at a concentration of 5 μM.

Sirolimus Flux Studies. HCT-8 and Caco-2 epithelial cell monolayers were used to examine the P-gp-mediated transport of sirolimus. Sirolimus was present at 0.1 μM which is 6- to 10-fold lower than the IC_{50} for inhibition of P-gp by this compound. Data from two experiments with HCT-8 cells and Caco-2 cells are presented in Table 2. The ratio of basolateral-to-apical (excretory) versus apical-to-basolateral (absorptive) flux was less than two in both these cell lines. Furthermore, cyclosporine, a well established P-gp inhibitor, failed to inhibit sirolimus flux in either cell line.

Caco-2 cells have also been observed to express members of the MRP gene family (Hirohashi et al., 2000; Chan et al., 2001). Both MRP1 and MRP3 actively transport substrates in the apical-to-basolateral direction, potentially masking P-gp-mediated basolateral-to-apical flux. To address this issue, sirolimus bidirectional flux across Caco-2 cell monolayers was evaluated in the presence of 1-chloro-2,4-dinitrobenzene (CDNB), an established MRP inhibitor (Evers et al., 1998). Addition of 50 μM CDNB to the medium did not change sirolimus flux in these cells; the ratio of basolateral-to-apical versus apical-to-basolateral sirolimus flux was 1.8 in the presence of the inhibitor versus 1.6 in the control. Combined, these data suggest that P-gp, MRP1, MRP2 (canalicular multispecific organic anion transporter), and MRP3 transporters do not mediate sirolimus flux in these cell lines.

Discussion

Consistent with previous reports, sirolimus was found to be extensively metabolized by CYP3A in vitro. Ketoconazole was an excellent inhibitor of sirolimus metabolism in human and rat liver microsomes, such that 1 μM ketoconazole reduced liver microsomal metabolism of sirolimus by $\geq$70% even though sirolimus was present at a saturating concentration (10 μM). Similar inhibition was observed for sirolimus metabolism by CYP3A4 Supersomes; however, ketoconazole was significantly less effective as an inhibitor of sirolimus metabolism by CYP3A5. The effect of ketoconazole was clearly observed in vivo, where oral coadministration of ketoconazole with Rapamune resulted in 5- to 6-fold increases in sirolimus levels in uninduced rats (Table 1). Consistent with studies in healthy volunteers (Benet, 2000), the large increases in sirolimus levels effected by ketoconazole were not accompanied by a decrease in variability, suggesting that issues beyond sirolimus metabolism (most likely physicochemical issues such as solubility and stability) are

TABLE 2
Sirolimus (0.1 μM) flux across HCT-8 cell monolayers
Flux was measured in the apical to basolateral (A to B, absorptive) and basolateral to apical (B to A, excretory) directions using a liquid chromatography-MS assay.

Experiment	Permeability (10^{-7} cm/s)[a]		
	A to B	B to A	B/A ratio
HCT-8			
Sirolimus alone, experiment 1	5.39 ± 1.4	7.35 ± 0.54	1.4
Sirolimus alone, experiment 2	2.46 ± 0.03	4.55 ± 0.51	1.8
+ Cyclosporine (5 μM)[b]	2.51 ± 0.08	3.57 ± 0.05	1.4
Caco-2			
Sirolimus alone, experiment 1	31.8 ± 4.3	45.0 ± 1.6	1.4
+ Cyclosporine (5 μM)[b]	51.8 ± 3.8	63.0 ± 5.8	1.2
Sirolimus alone, experiment 2	30.9 ± 4.1	54.5 ± 3.5	1.8

[a] Data are the mean ± S.D. of triplicate monolayers. A higher ratio indicates active extrusion.
[b] Flux ratios in the presence and absence of cyclosporine were compared using an unpaired Student's t test and were not statistically different.

significant contributors to the variability in sirolimus blood levels after oral absorption.

Despite being a substrate for CYP3A, sirolimus does not appear to be transported by P-gp. Significant polarized sirolimus flux was not observed in either direction across Caco-2 or HCT-8 cell monolayers (Table 2), suggesting that it does not undergo active transport in these systems. Low P-gp activity does not account for the findings of the current work, as we established that P-gp was present (by Western blot analysis) and activity was confirmed in both the HCT-8 and Caco-2 cell monolayers by conducting a R123 transport assay prior to conducting the sirolimus experiments. Similarly, the absence of polarized flux in the presence of the MRP inhibitor CDNB argues against a potential masking effect of MRP-mediated apical-to-basolateral transport in these monolayers. The results of the current work directly contrast the substantial polarized basolateral-to-apical (excretory) flux across Caco-2 monolayers reported by Crowe and Lemaire (1998) and the equally large apical-to-basolateral (absorptive) flux reported across the Caco-2 and HCT-8 monolayers used by Dias and Yatscoff (1994, 1996). The reasons for the dramatic differences in these results are unclear. Both previous studies measured radioactivity rather than absolute sirolimus levels, and it is conceivable that the differing results of those studies reflect some aberration in methodology. This was not the case in the current work, where intact sirolimus was measured using a specific liquid chromatography-MS assay. Consistent with published work (Arceci et al., 1992), sirolimus was a good inhibitor of P-gp, with an IC_{50} of 0.625 to 1.5 μM for inhibition of R123 flux across HCT-8 and Caco-2 cell monolayers.

The absence of P-gp-mediated sirolimus transport may, in part, explain the finding that TPGS did not improve sirolimus oral bioavailability in rats, despite being used at a dose that increased cyclosporine absorption 2- to 3-fold in identical experiments (Wacher et al., 2002). Since sirolimus does not appear to be a P-gp substrate, inhibition of intestinal P-gp by TPGS should have no effect on its absorption (compared with the TPGS effect on the established P-gp substrate cyclosporine). It is also conceivable that the failure of TPGS represents a negative interaction between TPGS and lipid-like excipients in the Rapamune formulation (phosphatidylcholine, propylene glycol, monodiglycerides, fatty acids, polysorbate 80). This argument is weakened by comparison to the cyclosporine data, where lipid-like excipients in the Sandimmune formulation (gelatin, glycerol, Labrafil M2125) did not impact TPGS at a similar 10-fold dilution in the saline gavage vehicle (Wacher et al., 2002). Moreover, the concentration of TPGS in the gavage solution was 5 mg/ml (0.5%), which is 100 to 500 times higher than the reported IC_{50} for inhibition of P-gp in vitro (0.001% in HCT-8 cells and 0.005% in Caco-2 cells; Dintaman and Silverman, 1999) and 25-fold higher than the TPGS critical micelle concentration [0.02% (w/w) = 0.2 mg/ml; Wu and Hopkins, 1999]. At this excess of TPGS, a modest excipient interaction should not have significantly impacted the activity of TPGS as a P-gp inhibitor and/or solubility enhancer. A detailed physicochemical evaluation of the effects of TPGS on sirolimus solubility was beyond the scope of the current work; however, it is clear that TPGS is not a useful bioavailability enhancer for coadministration with the Rapamune formulation.

In conclusion, the current work confirmed the impact of CYP3A on sirolimus oral bioavailability and determined that sirolimus is not transported by P-gp. The CYP3A inhibitor ketoconazole dramatically increased the oral bioavailability of sirolimus in uninduced rats; however, the solubilizing agent and P-gp inhibitor TPGS was ineffective. Routine coadministration of a safe, nonpharmacologically active CYP3A inhibitor may provide for lower sirolimus oral doses; however, the variability in sirolimus levels will also need to be addressed in an improved oral dosage form.

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