Metabolism and Transport of the Macrolide Immunosuppressant Sirolimus in the Small Intestine

ALFONSO LAMPEN, YUANCHAO ZHANG, INA HACKBARTH, LESLIE Z. BENET, KARL-FR. SEWING and UWE CHRISTIANS

Zentrumabteilung für Lebensmitteltoxikologie, Tierärztliche Hochschule Hannover, Hannover, Germany (A.L.); Department of Biopharmaceutical Sciences, School of Pharmacy, University of California at San Francisco, San Francisco, California (Y.Z., L.Z.B., U.C.); Institut für Allgemeine Pharmakologie, Medizinische Hochschule Hannover, Hannover, Germany (I.H., K.-F.R.S.); and Department of Cardiothoracic Surgery, Stanford University, Stanford, California (U.C.)

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ABSTRACT

Small intestinal metabolism and transport of sirolimus, a macrolide immunosuppressant with a low and highly variable oral bioavailability, were investigated using small intestinal microsomes and intestinal mucosa in the Ussing chamber. After incubation of sirolimus with human and pig small intestinal microsomes, five metabolites were detected using high performance liquid chromatography/electrospray-mass spectrometry: hydroxy, dihydroxy, trihydroxy, desmethyl and didesmethyl sirolimus. The same metabolites were generated by human liver microsomes and pig small intestinal mucosa in the Ussing chamber. Anti-CYP3A antibodies, as well as the specific CYP3A inhibitors troleandomycin and erythromycin, inhibited small intestinal metabolism of sirolimus, confirming that, as in the liver, CYP3A enzymes are responsible for sirolimus metabolism in the small intestine. Of 32 drugs tested, only known CYP3A substrates inhibited sirolimus intestinal metabolism with inhibitor constants (Ki) equal to those in human liver microsomes. The formation of hydroxy sirolimus by small intestinal microsomes isolated from 14 different patients ranged from 28 to 220 pmol·min⁻¹·mg⁻¹ microsomal protein. In the Ussing chamber, >99% of the sirolimus metabolites reentered the mucosa chamber against a sirolimus gradient, indicating active countertransport. Intestinal drug metabolism and countertransport into the gut lumen, drug interactions with CYP3A substrates and inhibitors in the small intestine and an 8-fold interindividual variability of the intestinal metabolite formation rate significantly contribute to the low and highly variable bioavailability of sirolimus.

The 31-member macrolide sirolimus (C₅₁H₇₉NO₁₃; molecular weight, 913.7 Da, fig. 1) is currently in phase III of its clinical development as an immunosuppressant after kidney transplantation and is under investigation as therapy after transplantation of other organs and for the treatment of autoimmune diseases. In the liver, sirolimus is metabolized by CYP3A enzymes (Sattler et al., 1992) to several hydroxylated and/or demethylated metabolites (Yatscoff et al., 1995; Christians et al., 1992; Wang et al., 1995; Streit et al., 1996a) of which the structures of 39-O-desmethyl, 16-O-desmethyl and 12-hydroxy sirolimus have been identified using ESI-MS/MS (Streit et al., 1996a). In addition, two degradation products have been identified after incubation of sirolimus with rat bile (Wang et al., 1994) and human liver microsomes (Streit et al., 1996a).

The immunosuppressants cyclosporin (Kolars et al., 1992; Lampen et al., 1996a) and tacrolimus (Lampen et al., 1995; 1996b), both CYP3A substrates in the liver, undergo metabolism in the small intestine, and the metabolites are mostly transported back into the gut lumen. Clinical studies suggest that intestinal metabolism and transport significantly contribute to the variable pharmacokinetics of cyclosporin and tacrolimus and that the intestine is the major site of drug interactions in patients (Hebert et al., 1992; Wu et al., 1995; Floren et al., 1997).

We hypothesize that sirolimus, as cyclosporin and tacrolimus, is metabolized in the small intestine. It was our objective to confirm the involvement of CYP3A in the intestinal metabolism of sirolimus, to compare the intestinal sirolimus metabolite patterns and drug interactions with those in the liver, to evaluate the interindividual variability of sirolimus metabolite formation in the small intestine and to study transport of sirolimus metabolites in the Ussing chamber.

Experimental Procedures

Materials and reagents. For analytical HPLC, we used a binary gradient Hewlett-Packard 1090A liquid chromatograph, equipped

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ABBREVIATIONS: CYP, cytochrome P450; ESI, electrospray ionization; MDR, multidrug resistance; HPLC, high performance liquid chromatography; MS, mass spectrometry.
with autosampler, autoinjector, 1040A diode array detector and an HP85B computer as data processor and integrator (Hewlett-Packard, Waldbronn, Germany). The 250 × 4-mm analytical HPLC column was filled with Hypersil C₈ material of 3-μm particle size (Shandon, Chadwick, UK).

The HPLC/MS system consisted of L-6000 and L6200 HPLC pumps and a 655 A40 autosampler (Merck, Darmstadt, Germany), connected through a 59887A electrospray interface equipped with an Iris Hexapole Ion Guide (Analytica of Branford, Branford, CT) to a 5989A mass spectrometer (Hewlett-Packard, Waldbronn, Germany). Data were recorded and analyzed using the MS-ChemStation Software (Version C.01.05, Hewlett Packard, Waldbronn, Germany). Analytical HPLC/MS columns were packed with Spherical C₁₈ material (3.9 × 150 mm, 5-μm particle size, Waters Millipore, Milford, MA). All solvents for HPLC and HPLC/MS analyses were purchased from Merck (Darmstadt, Germany) and were of HPLC quality.

Human small intestine samples for the isolation of microsomes were obtained from the Klinik für Abdominal- und Transplantationschirurgie (Medizinische Hochschule Hannover, Hannover, Germany). The collection of human small intestine samples for research was approved by the Ethical Committee of the Medizinische Hochschule Hannover.

Pig small intestine for the isolation of microsomes was obtained from the local slaughterhouse. Sirolimus was purchased from Sigma Chemical (St. Louis, MO).

The following drugs were, unless indicated otherwise, purchased from Sigma Chemical: acetaminophen, acetylsalicylic acid (Bayer, Leverkusen, Germany), amphotericin B, bromocryptine (Sandoz, Nürnberg, Germany), captorpril, corticosterone (Ciba-Geigy, Basle, Switzerland), cyclosporin (Sandoz, Basle, Switzerland), dexamethasone, diclofenac (Ciba-Geigy), erythromycin (Abbott Laboratories, Abbott Park, IL), ergotamine tartrate, diethyldithiocarbamate, disulfiram, ethinyl estradiol (Hoechst, Frankfurt, Germany), josamycin (Heinrich Mack, Illertissen, Germany), ketoconazole (Janssen, Neuss, Germany), lidocaine, meydylprednisonolone, miconazole (Hoffmann-La Roche, Basle, Switzerland), α-napthoflavone, naringine, nifedipine, omeprazole (Abbott Laboratories, Abbott Park, IL), phenytoin, prednisolone, progesterone (Glaxo, Hamburg, Germany), propranolol, quinidine, ranitidine, sulfaphenazole (Hoffmann-La Roche), trimethoprim, troleandomycin and verapamil.

Isocitric acid, NADP and isocitrate dehydrogenase were bought from Mannheim-Boehringer (Mannheim, Germany). 3-Methylcholanthrene was from Aldrich (Steinheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma Chemical (Deisenhofen, Germany) in the highest available purity.

Animals. Male Sprague-Dawley rats (Zentrales Tierlabor, Medizinische Hochschule Hannover, Hannover, Germany), weighing 220–250 g, were fed with a standard diet (Altromin, Lage, Germany) and had free access to drinking water. For the induction of various CYP enzymes, 3-methylcholanthrene and phenobarbital were given once daily by
intrapерitoneal injection for 3 days and dexamethasone once daily intraperitoneally for 2 days before the rats were killed.

**Small intestinal and liver microsomes.** Rat, pig and human enterocytes were isolated according to the methods described by Porteous et al. (1979) and Pinkus (1981). Microsomes were isolated by the differential centrifugation procedure as described by Guengerich (1982) with the modifications that a 0.1 M phosphate buffer (pH 7.4) was used instead of a Tris buffer and the ultra centrifugation steps (100,000 × g) were reduced to 45 min. Protein concentrations were measured using the bicinchoninic acid method (Smith et al., 1985) and a bovine serum albumin standard curve.

The protein concentrations of the microsomal suspensions were adjusted with 0.1 M phosphate buffer, pH 7.4. CYP concentrations were determined using the method described by Omura and Sato (1964) according to the protocol of Estabrook and Werringloer (1978).

**In vitro metabolism of sirolimus and sample preparation.** Ten microliters of 1 g/liter sirolimus in acetonitrile/sulfuric acid, pH 3 (75:25, v/v), were added to 1 ml of small intestinal microsomes (1.5 g of protein/liter). The resulting final sirolimus concentration was in the linear range of microsomal sirolimus metabolism. Ten microliters of vehicle acetonitrile/sulfuric acid, pH 3 (75:25, v/v), was added to the controls. In all assays, the final acetonitrile concentration was <0.5%, and acetonitrile concentrations up to 2.5% did not significantly inhibit sirolimus metabolism. The reaction was started by the addition of 0.5 ml of an NADPH generating system consisting of 2 mM EDTA, 10 mM MgCl₂, 84 mM NADP, 18 mM isocitric acid and 667 μl/iter isocitrate dehydrogenase. The reaction mixture was incubated for 40 min at 37°C under aerobic conditions. The reaction was stopped by protein precipitation with 0.5 ml of acetonitrile. The samples were centrifuged at 2500 × g for 2 min. The supernatant was drawn (vacuum: ~7 mm Hg) through 3-ml glass extraction columns (Kranich, Göttingen, Germany) filled with C₈ material of 25- to 40-μm particle size (LiChroPrep, Merck, Darmstadt, Germany), which had previously been washed with 3 ml of acetonitrile and 3 ml of sulfuric acid (pH 3.0). Then, 3 ml of methanol/sulfuric acid (pH 3.0) 50:50 v/v and 0.5 ml of hexane were drawn through the columns. These were subsequently dried by drawing air through the columns for 3 min. The extraction columns were set into 10-ml centrifuge tubes. Sirolimus and its metabolites were eluted using 1.5 ml of dichloromethane. Dichloromethane was evaporated at 40°C under a stream of nitrogen. Sirolimus and its metabolites were eluted using 1.5 ml of dichloromethane. Dichloromethane was evaporated at 40°C under aerobic conditions. The reaction was stopped immediately by the addition of 0.5 ml of acetonitrile.

In addition to the calibration samples, two quality control samples were run every 10 test samples. Sirolimus (1 or 10 μg) was added to 1 ml of the microsomal suspension (1.5 g/liter protein) and 0.5 ml of an NADPH generating system. The reaction was stopped at once by the addition of 0.5 ml acetonitrile. Quality control samples were randomly distributed and extracted among the study samples.

The day-to-day variability was 14.3%, and the detection limit of the HPLC assay was 50 μg/liter for sirolimus. The recovery of sirolimus and its metabolite from the microsomal suspensions ranged from 66% to 89%. The sirolimus calibration curve was linear up to ≥25 mg/liter (r = .99). None of the drugs tested interfered with the HPLC detection of sirolimus or its metabolites.

**HPLC/ESI-MS of sirolimus and its metabolites.** For HPLC/ESI-MS analysis, the extraction procedure for sirolimus and its metabolites was modified. After incubation, the reaction was stopped by the addition of 2 ml methanol/saturated zinc sulfate in water (50:50 v/v). 28-O-Acetyl sirolimus (10 μl of 1 mM in acetonitrile/ sulfuric acid, pH 3.0, 75:25 v/v), synthesized as described by Streit et al. (1996b), was added as internal standard. After centrifugation at 2500 × g, the supernatant was drawn (vacuum: ~7 mm Hg) through 3-ml extraction columns (Recipe, Munich, Germany) filled with C₈ material, which had been primed with 3 ml of acetonitrile and 1.5 ml of water. The samples were washed on the column with 3 ml of water. These were subsequently dried by drawing air for 3 min. The extraction columns were set into 10-ml centrifuge tubes. Sirolimus and its metabolites were eluted by centrifugation of 400 μl of acetonitrile/0.1% formic acid (90:10 v/v) through the columns (800 × g, 2 min).

Then, 25 μl of the extract was injected into the HPLC/MS system.

Sirolimus and its metabolites were eluted from the analytical column using the following parameters: mobile phase, methanol/0.1% formic acid (90:10 v/v), isocratic elution, flow rate, 0.5 ml/min, and column temperature, 35°C. The electrospray source was set to the following parameters: VCap, −5000 V; VEnd, −4500 V; VCy₁, −6000 V; capillary exit voltage, 200 V. The drying gas was adjusted to a value of 42 and heated to 350°C. The needle spray gas pressure was 80 psi. The quadrupole was heated to 120°C. The mass spectrometer was run in the positive ion mode; the multiplier voltage was set to 2740 V, and the X-ray voltage was set to 10,000 V. Because under these conditions the sodium adducts gave the strongest signals, the mass spectrometer was focused on the following ions ([M + Na⁺]⁺): m/z = 908 (didesmethyl sirolimus), m/z = 922 (desmethyl sirolimus), m/z = 936 (sirolimus), m/z = 952 (hydroxy sirolimus), m/z = 968 (dihydroxy sirolimus), m/z = 978 (internal standard 28-O-acetyl sirolimus). Quantification of hydroxy sirolimus did not interfere with sirolimus degradation products such as seco-rapamycin that are generated during incubation of sirolimus with microsomes (Streit et al., 1996a, Wang et al., 1994).

For quality control during the study, two precision and calibration control samples were run for every six samples. The calibration curve consisted of seven data points at concentrations of 0, 2.5, 1, 1.5, 2.5, 25 and 100 μg/liter for sirolimus and its metabolite hydroxy sirolimus and was linear from 0.25 to 100 μg/liter (r² = .98). Interassay (day-to-day) variability was 9.6% (n = 32). Quantification of the other metabolites was based on the assumption that the signal intensity of the respective molecular ions equaled those of sirolimus, hydroxy sirolimus or the internal standard, which were not different from each other.

**Isolation and identification of sirolimus metabolites.** Isolated metabolites were used as standards for validation of the HPLC and HPLC/ESI-MS assays. Hydroxy and 39-O-desmethyl sirolimus were isolated after metabolism of sirolimus by human liver microsomes according to the method described by Christians et al. (1992). The structures of the isolated metabolites were identified using HPLC/MS/MS in combination with collision induced dissociation and analysis of the fragmentation pattern (Streit et al., 1996a).

**Determination of Kₘ and Vₘₐₓ.** To 1 ml of the microsomal suspension (1.5 g/liter) protein, 0 (the addition of drug-free vehicle), 1.5, 3.75, 7.5, 15, 25 and 50 μM sirolimus (n = 5 for each sirolimus
samples taken from equivalent anatomical locations were included in the study. Eleven nanomoles of sirolimus were added to 1 ml of human small intestinal microsomes (1.5 g/liter) and 0.5 ml of NADPH generating system. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 500 μl of acetonitrile. The size of individual intestinal samples allowed only for comparison at a single sirolimus dose and not for determination of individual $K_m$ and $V_{max}$ values. The metabolism rate for individual microsomal preparations ($n = 3$ for each preparation) was determined, distribution statistics were calculated using the UNIVARIATE procedure and CYP3A activities of male and female patients were compared using the NPAR1WAY procedure (option Wilcoxon) of the SAS statistics program (version 6.05; SAS Institute).

**Ussing diffusion chamber.** Samples of the proximal duodenum of the pig gastrointestinal tract were obtained from the local slaughterhouse. The samples were rinsed with 1.32% NaCl and immediately stored in ice-cold Krebs-Henseleit buffer (pH 7.4) saturated with carbogen (95% O$_2$/5% CO$_2$). The lamina mucosa was stripped from the lamina muscularis and as far as possible from the lamina serosa. The Ussing chamber was set up as previously described (Lampen et al., 1996b). Under these conditions, the mucosa stabilized within 30 min. The conductivity was consistently ~20 mS/cm K$^+$. The system was stable for ≥8 hr. Sirolimus was added 45 min after setup of the Ussing chamber. If not stated otherwise, sirolimus dissolved in acetonitrile/water (pH 3, 75:25 v/v) was added to the mucosa chamber resulting in a final concentration of 10 μM and was incubated in the Ussing chamber for 4 hr.

**Results**

**Metabolism of sirolimus by human small intestinal microsomes.** After incubation of sirolimus with human intestinal microsomes for 1 hr, 60% was metabolized (fig. 2). The major metabolites generated and detected in the

![Fig. 2. Time-dependent generation of sirolimus metabolites by human small intestinal microsomes. Human small intestinal microsomes (1.5 g/liter protein in 0.1 M phosphate buffer) were incubated with 0.5 ml NADPH generating system and 10 μM sirolimus. Sirolimus metabolites were quantified using HPLC. A, Kinetics of sirolimus. B, Kinetics of 12-hydroxy and 39-O-desmethyl sirolimus. Concentrations are given as mean ± S.D. (n = 5).](image-url)
HPLC/UV chromatograms were 39-O-desmethyl and 12-hydroxy sirolimus, which reached their maximum concentrations at 40 min. The following sirolimus metabolites were generated by human small intestinal microsomes and detected by HPLC/ESI-MS: hydroxy, desmethyl, dihydroxy and didesmethyl sirolimus. The metabolite pattern was similar to that observed after incubation of sirolimus with pig small intestinal microsomes or human liver microsomes. Interspecies comparison of the small intestinal metabolism of sirolimus in rat, dog, human and pig is shown in Table 1. Analysis of variance combined with Duncan grouping showed no difference among dog, pig and human. However, rat small intestine metabolized sirolimus significantly slower than the other three species (P < .04). The \( K_{m} \) and \( V_{max} \) values for the formation of hydroxy sirolimus in pig small intestinal microsomes were similar to those in human small intestinal microsomes: human small intestinal microsomes: \( K_{m} \), 5.8 \( \pm \) 2.3 \( \mu \)M, \( V_{max} \), 89 \( \pm \) 18.4 pmol of hydroxy sirolimus\( \cdot \)min\(^{-1} \cdot \)mg\(^{-1} \) protein (mean \( \pm \) S.D., \( n \) = 5), and pig small intestine microsomes: \( K_{m} \), 6.2 \( \pm \) 2.1 \( \mu \)M, \( V_{max} \), 92 \( \pm \) 31 pmol\( \cdot \)min\(^{-1} \cdot \)mg\(^{-1} \) protein (\( n \) = 10).

**Identification of the CYP enzymes responsible for sirolimus metabolism.** Preincubation of human small intestinal microsomes with 250 \( \mu \)M troleandomycin significantly reduced generation of 12-hydroxy sirolimus by 75% (fig. 3A). The addition of 250 \( \mu \)M erythromycin reduced formation of 12-hydroxy sirolimus by 62%.

Only anti-CYP3A inhibited the formation of 12-hydroxy sirolimus, whereas anti-CYP2E1, anti-CYP2C and anti-CYP1A did not have any inhibitory effects. At a concentration of 10 mg of CYP3A antibody/mg of protein, the formation of hydroxy sirolimus was reduced by 69% (fig. 3B).

In comparison with microsomes from untreated rats, the specific cytochrome CYP3A inducer dexamethasone increased sirolimus metabolite formation. When microsomes of dexamethasone induced rats were used, the formation rate of 12-hydroxy sirolimus and 39-O-desmethyl sirolimus was 8.7- and 7.2-fold higher than using microsomes from untreated controls, respectively. Representative HPLC/UV chromatograms after incubation of sirolimus with microsomes isolated from rats that had been treated with various CYP inducers are shown in figure 4. The other CYP enzyme inducers included in the study, phenobarbital and 3-methylcholanthrene, failed to affect sirolimus metabolism.

In intestinal microsomes isolated from 14 different patients (see Interindividual Variability), formation of 12-hydroxy sirolimus was significantly correlated with the CYP3A-mediated formation of 13-O-desmethyl tacrolimus (Lampen et al., 1995) (\( r \) = .98, P < .001).

Based on these results, it was concluded that CYP 3A enzymes are responsible for hydroxylation of sirolimus in the small intestine.

**In vitro drug interactions with the metabolism of sirolimus in pig small intestine microsomes.** Of the 32 xenobiotes tested, the following 19 did not affect the *in vitro* metabolism of sirolimus by pig small intestinal microsomes: acetaminophen, acetylsalicylic acid, amphotericin B, \( \alpha \)-naphthoflavone, captopril, cimetidine, diclofenac, diethyldithiocarbamate, disulfiram, lidocaine, naringinine, omeprazole, phenytoin, progesterone, propranolol, quinidine, ranitidine, sulfaphenazon and trimethoprim.

The \( K_{i} \) values of the inhibitors of *in vitro* sirolimus metab-

**Table 1**

Comparison of 12-hydroxy and 39-O-desmethyl sirolimus formation by microsomes isolated from the proximal small intestine of different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Metabolite formation rate</th>
<th>12-Hydroxy sirolimus</th>
<th>39-O-Desmethyl sirolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min ( \cdot )mg(^{-1} )</td>
<td>( \mu )M</td>
<td>( \mu )M</td>
</tr>
<tr>
<td>Rat (( n = 5 ))</td>
<td>32.1 ( \pm ) 13.8</td>
<td>0.34 ( \pm ) 0.09</td>
<td>0.18 ( \pm ) 0.05</td>
</tr>
<tr>
<td>Dog (( n = 3 ))</td>
<td>73.9 ( \pm ) 28.5</td>
<td>0.76 ( \pm ) 0.34</td>
<td>0.28 ( \pm ) 0.09</td>
</tr>
<tr>
<td>Pig (( n = 10 ))</td>
<td>77.2 ( \pm ) 34.7</td>
<td>0.97 ( \pm ) 0.43</td>
<td>0.32 ( \pm ) 0.10</td>
</tr>
<tr>
<td>Human (( n = 14 ))</td>
<td>97.7 ( \pm ) 61.3</td>
<td>1.12 ( \pm ) 0.36</td>
<td>0.54 ( \pm ) 0.13</td>
</tr>
</tbody>
</table>
Intestinal Metabolism of Sirolimus

Inhibition constants ($K_i$) and mechanism of inhibition of drugs interacting with the in vitro formation of 12-hydroxy sirolimus by human liver microsomes and pig small intestinal microsomes

To determine the $K_i$ values, the individual Michaelis-Menten kinetics (n = 5) were fitted and $K_i$ was determined using a secondary plot with $K_i/V_{max}$ vs. the inhibitor concentration and linear regression analysis. The $K_i$ are given as mean ± S.D.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Pig small intestine</th>
<th>$K_i$ (μmol/liter)</th>
<th>Human liver</th>
<th>$K_i$ (μmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromocryptine</td>
<td>50 ± 2.4</td>
<td>-</td>
<td>65 ± 2.7</td>
<td>-</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>18 ± 2.7</td>
<td>+</td>
<td>35 ± 3.5</td>
<td>+</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>110 ± 12.9</td>
<td>-</td>
<td>125 ± 19.8</td>
<td>-</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>35 ± 7.7</td>
<td>+</td>
<td>50 ± 7.5</td>
<td>+</td>
</tr>
<tr>
<td>Ergotamine</td>
<td>55 ± 8.9</td>
<td>-</td>
<td>40 ± 5.8</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>23 ± 5.7</td>
<td>+</td>
<td>85 ± 7.3</td>
<td>+</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td>160 ± 11.5</td>
<td>-</td>
<td>125 ± 22.4</td>
<td>-</td>
</tr>
<tr>
<td>Josphycin</td>
<td>25 ± 4.5</td>
<td>-</td>
<td>35 ± 3.7</td>
<td>-</td>
</tr>
<tr>
<td>Ketocanazole</td>
<td>15 ± 3.1</td>
<td>-</td>
<td>25 ± 5.3</td>
<td>-</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>80 ± 12</td>
<td>-</td>
<td>90 ± 11</td>
<td>-</td>
</tr>
<tr>
<td>Miconazole</td>
<td>20 ± 4.1</td>
<td>-</td>
<td>24 ± 3.8</td>
<td>-</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>5 ± 1.7</td>
<td>+</td>
<td>15 ± 4.3</td>
<td>+</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>33 ± 6.2</td>
<td>+</td>
<td>25 ± 5.1</td>
<td>+</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>15 ± 2.5</td>
<td>-</td>
<td>20 ± 4.6</td>
<td>-</td>
</tr>
<tr>
<td>Verapamil</td>
<td>53 ± 8.6</td>
<td>-</td>
<td>40 ± 8.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3
Interindividual variability of the 12-hydroxy sirolimus formation by human small intestinal microsomes

Human small intestinal samples were taken from patients undergoing gut surgery and from anatomically equivalent locations of the duodenum. Samples were included in the study only when the patients did not take drugs known to be CYP3A inhibitors or inducers. Data are presented as mean ± S.D. of three determinations for each sample.

<table>
<thead>
<tr>
<th>Formation rate of 12-hydroxy sirolimus</th>
<th>nmol/min mg⁻¹</th>
<th>1/mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>160.1 ± 25.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>219.8 ± 42.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>74.7 ± 13.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>179.4 ± 28.9</td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>158.7 ± 61.2</td>
<td></td>
</tr>
<tr>
<td>Male patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>43.4 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>27.6 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>57.9 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>104.7 ± 17.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>50.2 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>34.7 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>89.7 ± 17.2</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>150.8 ± 21.6</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>43.4 ± 7.7</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>132.0 ± 17.3</td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>73.5 ± 43.2</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The present study showed that sirolimus is metabolized in the small intestine, as in the liver, by CYP3A enzymes resulting in a metabolite pattern equal to that generated in the liver. Metabolism of sirolimus in the small intestine varied interindividually ~8-fold. Drugs that are CYP3A substrates interfered with sirolimus metabolism in the small intestine and the inhibition constants ($K_i$) were similar to those in the liver. Most of the sirolimus metabolites formed in the small...
intestinal mucosa were transported back through the luminal membranes of gut mucosa cells.

Two analytical methods were used to quantify sirolimus and its metabolites: HPLC/UV and HPLC/MS. 39-O-Desmethyl sirolimus and 12-hydroxy sirolimus were separated from the other metabolites, and their structures were confirmed by HPLC/MS/MS analysis as described previously (Streit et al., 1996a). All other metabolite peaks represented mixtures of different metabolites (Christians et al., 1992). The HPLC/MS method used (Streit et al., 1996b) allowed for separate quantification of metabolites with different m/z values and the same HPLC retention times but was not able to differentiate between metabolites with the same retention time and m/z values (Streit et al., 1996b). It was not possible to assign the exact structure of the metabolites after HPLC/MS analysis. Therefore, a metabolite determined by HPLC/MS measurement may represent different metabolites with the same modification such as hydroxylation or demethylation but in different positions. Although the HPLC/MS allowed for quantitation of all known and several yet unidentified metabolites such as dihydroxy sirolimus, and desmethyl sirolimus, it could not be excluded that more polar metabolites were lost during extraction and/or missed by HPLC/MS analysis.

It has to be taken into account that only apparent enzyme kinetic parameters were determined for the following reason: Microsomes do not represent an isolated enzyme but a mixture of different enzymes and more than one enzyme of the CYP3A family may be involved in sirolimus metabolism. Kolars et al. (1994) reported that in addition to CYP3A4, CYP3A5 is expressed in significant amounts in the small intestine. In addition, most of the inhibitors were also CYP3A substrates and metabolized during the incubation period.

The highest CYP3A concentration in the gastrointestinal tract is present in the duodenum and declines in the following order: duodenum > jejunum > ileum > colon (Kolars et al., 1994; Lampen et al., 1996b). To avoid interference with variability resulting from the different origins of small intestinal samples, it was essential to collect samples from equivalent anatomical positions of the duodenum. Duodenal samples were chosen since they have the highest CYP3A concentration and the proximal small intestine is where sirolimus is predominantly absorbed.

Since the amount of small intestinal microsomes required to study drug interactions was not available from humans, pig small intestinal microsomes were used. However, it is well known that the metabolite patterns of other drugs in pig, such as midazolam, which is also a CYP3A substrate, differ significantly from those in humans (Gorski et al., 1994; Ochs et al., 1987). Therefore, the validity of the use of pig microsomes was thoroughly evaluated and justified by the following results: in Western blot analysis, pig CYP enzymes corresponding to human CYP3A enzymes were detected using a human CYP3A antibody (Lampen et al., 1995), the sirolimus metabolite pattern as checked by HPLC/ESI-MS analysis equaled those generated by human intestinal and liver microsomes, and the $K_m$ and $V_{max}$ values for formation of 12-hydroxy sirolimus were similar to those in human liver microsomes. Retrospectively, the validity of the pig small intestinal microsome model was confirmed by obtaining $K_i$ values similar to those determined in human liver microsomes.

To date, drug interactions with sirolimus metabolites have not been studied. In our study, the same drugs that interact with the in vitro metabolism of the immunosuppressants and CYP3A substrates cyclosporin (Pichard et al., 1990) and tacrolimus (Christians et al., 1996) also interacted with the in vitro metabolism of sirolimus. Thus, it can be expected that the same drugs as described for cyclosporin (Yee and McGuire, 1990) and tacrolimus (Peters et al., 1993) will affect sirolimus pharmacokinetics in patients. Although the liver has always been regarded as the principal site of drug interactions, most recent in vitro and clinical studies strongly indicated that small intestinal metabolism and transport is responsible for the low and variable oral bioavailability of many drugs that are CYP3A substrates (Benet et al., 1996;
Wacher et al., 1995) and a major site of drug interactions (Hebert et al., 1992; Wu et al., 1995; Floren et al., 1997). Because CYP3A enzymes are responsible for sirolimus metabolism in the small intestine, all drugs that inhibited CYP3A-dependent metabolism in the liver, as expected, also inhibited sirolimus metabolism in the small intestine. In the small intestine, the $K_v$ values of most inhibitors equaled those in the liver. Xenobiotics, which are known specific inhibitors of CYP enzymes other than CYP3A such as α-naphthoflavone (CYP1A1; Chang et al., 1994), sulfaphenazole (CYP2C; Guengerich et al., 1992), quinidine, propranolol (both 2D6; Birkett et al., 1993), diethylthiocarbamate and disulfiram (both CYP2E1; Chang et al., 1994; Bargetzi et al., 1989), failed to inhibit hydroxylation of sirolimus in the liver, as well as in the small intestine, thus excluding involvement of these respective CYP enzymes.

These in vitro results cannot be extrapolated to clinical drug interactions without restrictions. In general, if appropriate concentrations of the drugs are used, lack of an in vitro drug interaction is reassuring and can generally eliminate any contribution of metabolites to sirolimus toxicity is available. Sirolimus doses in clinical studies ranged from 0.5 to 6.5 mg/m$^2$ (Zimmerman and Kahn, 1997). Therefore, sirolimus concentrations reached locally at the site of absorption in the small intestine can be expected to be in the concentration range of our in vitro drug interaction assays (1.5–50 μM). The concentration of sirolimus in the liver of patients is yet unknown. Maximum blood concentrations do usually not exceed 0.1 μM. However, it has been shown that sirolimus concentrations in portal venous blood are several-fold higher than systemic blood concentrations (Shapiro et al., 1997) and that in the rat, depending on the sirolimus dose, liver tissue concentrations are up to 20-fold higher than blood concentrations (Napoli et al., 1997).

Because sirolimus is currently under development as an immunosuppressant after kidney transplantation in combination with cyclosporin, the significant inhibition of liver and intestinal sirolimus metabolism by cyclosporin is of particular importance. According to the results of the present study, it can be expected that cyclosporin will significantly increase the oral bioavailability of sirolimus. This hypothesis is supported by a study from Stepkowski et al. (1996). These authors demonstrated higher sirolimus concentrations in blood of rats after oral administration with, than without, coadministration of cyclosporin, resulting in better graft survival. However, we demonstrated in the present study that sirolimus is significantly slower metabolized in the rat than in humans. On the basis of these data, it can be hypothesized that in patients, the impact of cyclosporin on the oral bioavailability of sirolimus is even greater than that reported for the rat.

The 8-fold variability of sirolimus metabolite formation in 14 patient samples can be expected to significantly contribute to the high interindividual variability of sirolimus oral bioavailability. The variability is in the same range as that reported for the intestinal metabolism of tacrolimus (Lampen et al., 1995) and cyclosporin (Lampen et al., 1996a) as well as for CYP3A expression in the small intestine (Lown et al., 1995). As observed for cyclosporin (Lampen et al., 1996a) and tacrolimus (Lampen et al., 1995), in comparison with male small intestinal microsomes, sirolimus was more rapidly metabolized by the small intestinal microsomes isolated from female patients. In all three studies, microsomes from the same tissue samples were used. Due to the small number of female patients included in the study, it is impossible to decide whether this observation is of significance.

As reported for tacrolimus (Lampen et al., 1996b), sirolimus metabolites are mainly found in the luminal compartment of the Ussing chamber. Because of the high sirolimus concentration in the lumen chamber, active transport can be anticipated. There is strong evidence that in addition to CYP3A enzymes, anti-transport by P-glycoprotein and other members of the ATP-binding cassette (ABC) transporter family into the gut lumen reduce the oral bioavailability of drugs that are CYP3A substrates (Benet et al., 1996; Wacher et al., 1995). There is a strong overlap of substrate specificities of CYP3A enzymes and P-glycoprotein (Wacher et al., 1995, 1996).

The present study indicates that intestinal metabolism and transport, interindividual variability of intestinal metabolism and drug interactions at the level of intestinal metabolism and transport significantly contribute to the low and variable oral bioavailability of sirolimus. On the basis of the present study, controlled inhibition of intestinal countertransport and/or metabolism, as discussed by Benet et al. (1996), can be expected to improve oral bioavailability and pharmacokinetic variability of sirolimus.

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Send reprint requests to: Uwe Christians, MD., Ph.D., Department of Biopharmacological Sciences, School of Pharmacy, University at San Francisco, 513 Parranossa Ave., Room 8-334, San Francisco, CA 94143-0448. E-mail: uwex@itsa.ucsf.edu