METABOLISM OF THE IMMUNOSUPPRESSANT TACROLIMUS IN THE SMALL INTESTINE: CYTOCHROME P450, DRUG INTERACTIONS, AND INTERINDIVIDUAL VARIABILITY

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ABSTRACT:

The small intestinal metabolism of tacrolimus, which is used as an immunosuppressant in transplantation medicine, was investigated in this study. Tacrolimus was metabolized in vitro by isolated human, pig, and rat small intestinal microsomes. The metabolites generated were identified by HPLC/MS. Tacrolimus and its metabolites were quantified using specific CYP antibodies and inhibitors. For characterization of the interindividual variability, microsomes were isolated from small intestinal samples of patients who had undergone resection for various reasons. In an in vitro model using pig small intestinal microsomes, 32 drugs were analyzed for their interactions with tacrolimus metabolism. After incubation with human, rat, and pig small intestinal microsomes, the metabolites 13-O-demethyl and 13,15-O-demethyl tacrolimus were identified. The metabolism of tacrolimus by human small intestine was inhibited by anti-CYP3A, troleandomycin, and erythromycin, indicating that, as in the liver, CYP3A enzymes are the major enzymes for tacrolimus metabolism in the human small intestine.

Metabolism of tacrolimus by small intestinal microsomes isolated from 14 different patients varied between 24 and 110 pmol/13-O-demethyl tacrolimus/min/mg microsomal protein, with a mean ± SD of 54.2 ± 29.2 pmol/min/mg. Of 32 drugs tested, 15 were found to inhibit small intestinal tacrolimus metabolism: bromocryptine, corticosterone, cyclosporine, dexamethasone, ergotamine, erythromycin, ethinyl estradiol, jasamine, ketoconazole, nifedipine, omeprazole, progesterone, rapamycin, troleandomycin, and verapamil. All of these drugs inhibited tacrolimus metabolism by human liver microsomes as well. It is concluded that tacrolimus is metabolized by cytochrome CYP3A enzymes in the small intestine. The rate of the CYP3A enzymatic activities varies about 5 times from patient to patient, and drugs that interfere with the in vitro metabolism of tacrolimus in the liver also inhibit its small intestinal metabolism.

Tacrolimus (FK506, Fujisawa, Osaka, Japan) is isolated from Streptomyces tsukubaensis (1, 2) and has a 23-member macrolide lactone structure (C_{44}H_{68}NO_{12}) with a molecular weight of 803.5 Da (fig. 1). It has proven to be a potent immunosuppressant in transplantation medicine (3, 4), for therapy of acute rejection (5), and in treatment of different immune diseases (6–8).

The structures of nine tacrolimus metabolites generated by liver microsomes of different species have been identified to date (9–11). The reactions involved in the metabolism of tacrolimus are hydroxylation at C(12), C(19), and the C(36) and C(37) vinyl group, as well as demethylation at C(13), C(15), and C(31). In addition, demethylation at C(13) and hydroxylation at C(12) affect the structure of the macroclide ring (9). Demethylated, double demethylated, hydroxylated, and double hydroxylated tacrolimus metabolites, as well as metabolites that are both hydroxylated and demethylated, have been detected in blood, bile, and urine of liver graft recipients (12, 13). The demethylated metabolites exhibit an immunosuppressive activity up to 70% of that of the parent compound (9). Tacrolimus is mainly excreted via bile in the form of several metabolites (14). Enzymes of the CYP 3A subfamily are the major CYP enzymes found in the proximal small intestine (15, 16). It has been hypothesized that interactions with the small intestinal CYP enzymes may affect pharmacokinetics and absorption of drugs, and may be responsible for the low and highly variable oral bioavailability of most CYP3A substrates, such as cyclosporine (17–20).

Enzymes of the CYP3A subfamily have been identified as being responsible for the O-demethylation of tacrolimus in the liver (21–23). It was shown that known CYP3A substrates, such as cyclosporine, nifedipine, and erythromycin, inhibit the in vitro metabolism of tacrolimus by isolated human liver microsomes (24). Tacrolimus has a low (mean 27%) and highly variable oral bioavailability (8–69%) (25), and it can be hypothesized that intestinal metabolism might be involved.

Materials and Methods

Materials and Reagents. For analytical HPLC, a binary gradient Hewlett-Packard 1090A liquid chromatograph, equipped with autosampler, autoinjec-

1 Abbreviations used are: CYP, cytochrome P450; K_i, inhibition constant; K_M, Michaelis constant; V_{max}, maximum initial velocity.
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Hewlett-Packard). Analytical HPLC/IMS columns were packed with Finigan (Steinheim, Germany). All other chemicals were purchased from Merck or local suppliers.

Small Intestinal and Liver Microsomes. Rat, pig, and human enterocytes were isolated according to the methods described by Porteous et al. (28) and Pinkus (29). Microsomes were isolated by differential centrifugation according to Guengerich (30), with the modifications that a 0.1 M phosphate buffer (pH 7.4) was used instead of a Tris buffer and the times of the centrifugation steps (100,000g) were reduced to 45 min. The protein concentration was determined using the bicinchoninic acid method (31). The working solution consisted of bicinchoninic acid solution (Sigma Chemicals)/(4%), w/v) CuSO4·5H2O in water 98:2 (v/v). One hundred µl of working solution and a 100-µl sample were incubated for 10 min in the wells of a microtiter plate, and absorbance was measured at 550 nm in a plate reader. Protein concentrations were estimated from a bovine serum albumin standard curve. The protein concentration of the microsomal suspension was adjusted to 3 g/liter, with a 0.1 M phosphate buffer (pH 7.4). The CYP concentration was determined using the method described by Omura and Sato (32) following the protocol described by Estabrook and Werringloer (33).

The source of human liver samples, the isolation, and characterization of microsomes were described by Sattler et al. (22).

In Vitro Metabolism of Tacrolimus and Sample Preparation. Ten µl of 1 g/liter tacrolimus in acetonitrile/water (pH 3 (70/30, v/v)) was added to 1 ml of small intestinal microsomes (1.5 g protein/liter). The reaction was started by addition of 0.5 ml of an NADPH generating system consisting of 2 mM EDTA, 10 mM MgCl2, 0.84 mM NADPH, 18 mM isocitric acid, and 667 units/liter isocitrate dehydrogenase. The mixture was incubated for 20 min at 37°C under aerobic conditions. The reaction was stopped by adding 0.5 ml acetonitrile. The samples were centrifuged at 2500g for 2 min. The supernatant was drawn (vacuum: 7 mm Hg) through 3-mL glass extraction columns (Kranich, Gött-ingen, Germany) filled with C8 material of 25–40 µm particle size (LiChro- prep; Merck) that were previously washed with 3 ml acetonitrile and 3 ml sulfuric acid (pH 3.0). Three ml methanol/sulfuric acid (pH 3.0) 50/50, v/v) and 0.5 ml hexane were drawn through the columns. These were subsequently dried by drawing air through the columns for 3 min. The extraction columns were set in 10-ml centrifuge tubes. Tacrolimus and its metabolites were eluted using 1.5 ml dichloromethane. After dichloromethane had been evaporated at 56°C under a stream of nitrogen, tacrolimus and its metabolites were dissolved in 300 µl acetonitrile/sulfuric acid pH 3.0 (70/30, v/v). The samples were washed again with 0.5 ml hexane, and 125 µl were injected into the HPLC system.

Quantification of Tacrolimus and 13-O-demethyl Tacrolimus by HPLC. Tacrolimus and its metabolites were eluted from the 250 × 4 mm analytical column using a concave sulfuric acid (pH 3.0)/acetonitrile gradient—analysis time, 0 min; 42% acetonitrile—analysis time, 20 min; 48% acetonitrile—analysis time, 35 min; 57% acetonitrile—analysis time, 45 min; and 75% acetonitrile. The elution was followed by a column clean-up step washing the column with 95% acetonitrile for 5 min and reequilibration to the start conditions for 7 min. The flow was 0.7 ml/min, the column temperature was 75°C, and the UV detection wavelength was 205 nm.

13-O-demethyl tacrolimus was identified in the chromatograms using the retention time of the structurally identified material (vide infra). A standard sample containing 13-O-demethyl tacrolimus was run every 20 test samples. Tacrolimus and its 13-O-demethyl metabolite were quantified using external calibration curves. This was possible because tacrolimus and its metabolite proved to have similar UV spectral characteristics. Calibration curves were prepared as follows. Tacrolimus was added to 1 ml of a microsomal suspension (1.5 g/liter protein) and 0.5 ml NADPH generating system to result in the following final concentrations: 0, 0.25, 0.5, 0.75, 5, and 25 mg/liter with N = 5 for each concentration. The reaction was stopped immediately by addition of 0.5 ml acetonitrile. The calibration curve was randomly distributed among the test samples and a separate calibration curve for each drug tested was run.

In addition to the calibration samples, two quality control samples were run every 10 test samples. One µg or 10 µg tacrolimus were added to 1 ml of the microsomal suspension (1.5 g/liter protein) and 0.5 ml of a 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 12.1%, the detection limit of the HPLC assay was 50 µg/liter for tacrolimus, and the recovery of tacrolimus and its metabolite from the microsomal suspensions ranged from 65.5% to 89%. The tacrolimus calibration curve was linear up to at least 25 mg/liter (r = 0.99). Drug interferences with the HPLC assay were tested, and the results were previously described (12).
HPLC/MS of Tacrolimus and Its Metabolites. Samples were extracted as described for the HPLC assay, and 25 μl of the resulting extract was injected onto a Spherical C18 column (Waters Millipore). Tacrolimus and its metabolites were eluted isocratically with methanol/water (90/10, v/v) at a flow rate of 0.4 ml/min. The MS parameters were adjusted as previously described (12). For chemical ionization, methane (purity > 99.5%, Messer, Griesheim, Germany) was used as reagent gas, and negative ions were detected. For metabolite identification, the mass spectrometer was run in the scan mode and for quantification of tacrolimus and its metabolites in the selected ion mode and focused on the following masses: 775.7 amu (didemethyl tacrolimus), 789.7 amu (demethyl tacrolimus), 791.7 (demethyl-hydroxy tacrolimus), 803.7 amu (tacrolimus), 805.7 amu (demethyl-hydroxy tacrolimus), 807.7 amu (didemethyl-dihydroxy tacrolimus), 819.7 amu (hydroxy tacrolimus), and 845.7 amu (32-O-acetyl tacrolimus; internal standard).

For quality control during the study, two precision and calibration control samples were run for every six samples. The calibration curve comprised six data points at concentrations of 0, 0.5, 1, 1.5, 5, 25, and 100 μg/liter for tacrolimus and its metabolites 13-O-demethyl and 13,15-O-demethyl tacrolimus, and was linear from 0.2 μg/liter to 100 μg/liter (r = 0.98). Quantification of the other metabolites was based on the assumption that the signal intensity of the respective molecular ions equaled those of tacrolimus, 13-O-demethyl, 13,15-O-didemethyl tacrolimus, or the internal standard. These were not different from each other. The day-to-day variability was 11.1% for tacrolimus.

Isolation and Identification of Tacrolimus Metabolites. Isolated metabolites were used as standards for validation of the HPLC and HPLC/MS methods. Tacrolimus was isolated after metabolism of tacrolimus by human liver microsomes as described previously (34). The metabolites 13-O-demethyl, 15-O-demethyl, 31-O-demethyl, 12-hydroxy, and 13,15-O-demethyl tacrolimus were isolated using the method described by Iwasaki et al. (9, 11). The structures of the metabolites were identified by HPLC/electrospray/MS/MS and analysis of the fragmentation pattern. Because sufficient material of this metabolites was isolated, the structure of 13-O-demethyl tacrolimus was evaluated by one- and two-dimensional, homonuclear and heteronuclear NMR techniques (35). The identity of the metabolites was confirmed by assessment of their immunosuppressive activity and cross-reactivity with an antibody used for an ELISA assay for tacrolimus (9, 11). To identify the tacrolimus metabolites after metabolism by human, rat, and pig small intestinal microsomes, the fractions containing tacrolimus metabolites were isolated by preparative HPLC. The structures were identified by HPLC/electrospray/MS/MS (Taq700, Finnigan MAT, Bremen, Germany) and analysis of the fragmentation pattern. These were compared with those of the metabolite standards.

Immunoblot Analysis. Microsomal protein from each small intestinal sample was subjected to immunoblot analysis of the CYP pattern. Proteins were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels (32 g/ml for 2 hr) and then electrophoretically transferred to polyvinylidendifluorid membranes (Millipore, Eschborn, Germany). The membranes were blocked overnight in phosphate-buffered saline containing 3% bovine serum albumin and 1% Tween 20 (Sigma Chemicals). Membranes were incubated sequentially with a rabbit polyclonal antibody (36) and alkaline phosphatase-conjugated sheep anti-rabbit immunoglobulin G. Immunoreactive proteins were visualized by incubating the membranes in a solution containing 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium.

Chemical Inhibition of CYP 3A Activities. Troleandomycin is a selective inhibitor of CYP3A enzymes (37). Troleandomycin was dissolved in ethanol. Fifteen μl was added to 1 ml of human small intestine microsomes (1.5 g protein/liter of 0.1 M phosphate buffer, pH 7.4) and 50 μl NADPH. The final concentrations of troleandomycin in the assay ranged from 0 to 250 μM. The samples were preincubated for 20 min to inactive CYP3A enzymes. Then, 450 μl NADPH generating system and tacrolimus (final concentration in the assay: 20 μM) were added. After an incubation period of 20 min at 37°C, the reaction was halted using 500 μl acetonitrile. Erythromycin is a specific, competitive inhibitor of CYP3A enzymes (20). Tacrolimus (final concentration: 20 μM), erythromycin (final concentration: 0–250 μM), 1 ml of human small intestinal microsomes (1.5 g/L protein), and 0.5 ml of a NADPH generating system were incubated for 20 min at 37°C. The reaction was stopped by adding 500 μl acetonitrile.

FIG. 2. Time-dependent generation of tacrolimus metabolites.

Human small intestinal microsomes (1.5 g/liter of protein in 0.1 M phosphate buffer) were incubated with 0.5 ml NADPH generating system and 10 μM tacrolimus for various time periods. Tacrolimus and its metabolites were quantified by HPLC/MS. (A) Kinetics of tacrolimus. (B) Kinetics of the metabolites. All concentrations are given as means ± SEs (N = 5). ?, 13-O-demethyl-tacrolimus; V, didemethyl-hydroxy-tacrolimus; O, 13,15-didemethyl-tacrolimus; W, hydroxy-tacrolimus.

Immunoinhibition with CYP Antibodies. Rabbit anti-human CYP2E1, anti-human CYP3A, anti-human CYP2C, and anti-human CYP1A were prepared as described elsewhere (36, 38–40).

The antibodies or preimmune IgGs were preincubated (15 min for 37°C) with human small intestinal microsomes (0.3 g/liter protein) and 0.375 ml of a NADPH generating system. The reaction was started by the addition of tacrolimus at a final concentration of 8.3 μM in the assay and was terminated by the addition of 500 μl acetonitrile.
concentrations using analysis of variance (GLM procedure, SAS version 6.05; 0.5 ml of the NADPH generating system were added. The inhibitor was tested at different locations in the small intestine (42). To avoid this type of variation, only duodenal samples taken from equivalent locations were included in the study. Twelve nmol of tacrolimus was added to 1 ml human small intestinal microsomes (1.5 g/liter) in 0.1 M phosphate buffer (pH 7.4) and 0.5 ml acetonitrile. Distribution statistics were calculated using the univariate procedure, and the 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).

**Results**

**Metabolism of Tacrolimus in Human Small Intestinal Microsomes.** The time-dependent disappearance of tacrolimus and generation of its metabolites by human small intestinal microsomes is shown in fig. 2.

After incubation of tacrolimus with human small intestine microsomes, four metabolites were identified by HPLC/MS (fig. 3C): didemethyl tacrolimus (775.5 amu), demethyl tacrolimus (789.5 amu), didemethyl-hydroxy tacrolimus (791.5 amu), and hydroxy tacrolimus (819.5 amu). The metabolites were similar to those after incubation of tacrolimus with pig small intestine microsomes or human liver microsomes (fig. 3A and B).

**Drug Interactions.** Ten μl tacrolimus [1 g/liter in acetonitrile/sulfuric acid, pH 3 (70/30, v/v)] and the drugs tested (concentrations *vide infra*) were added to 1 ml pig small intestinal or human liver microsomes (1.5 g/liter protein). The drugs tested for interaction with the tacrolimus metabolism were dissolved in ethanol, except for omeprazole (methanol), cimetidine (acetonitrile/water, pH 3; 70/30, v/v), ergotamine (methanol/water, 50/50, v/v), ethinyl estradiol (dimethylsulfoxide), bromocrytine (methanol), and amphotericin B (dimethylsulfoxide). They were added to the metabolism mixtures to yield the following final concentrations: 0, 0.1, 1, 10, 100, 250, 500, and 1000 μmol/liter (N = 5 for each concentration).

Formation of 13-O-demethyl tacrolimus was compared at the different inhibitor concentrations using analysis of variance (GLM procedure, SAS version 6.05; SAS Institute, Cary, NC). When a significant inhibition was detected (p < 0.05), the $K_I$ was determined on the basis of a Michaelis-Menten kinetics. To 1 ml of the microsomal suspension (1.5 g/liter) protein, 0, 1.5, 3.75, 7.5, 15, 25, and 50 mg/liter tacrolimus (N = 5 for each tacrolimus concentration), the inhibitor, and 0.5 ml of the NADPH generating system were added. The inhibitor was tested at three different concentrations: 0 μmol/liter and two concentrations between the half-maximal inhibition concentration and 300 μmol/liter. The samples were incubated, extracted, and quantified as described herein. Data were fitted and $K_I$ and $V_{max}$ calculated using the Enzfitter software (version 1.03, Elsevier-Biosoft, Cambridge, UK). $K_I$ was determined using a secondary plot with $K_I/V_{max}$ vs. the inhibitor concentration and linear regression analysis. The type of inhibition was determined from a Lineweaver-Burk plot.

**Interindividual Variability.** Small intestine samples were collected from 14 patients undergoing intestinal surgery for various reasons. Patients did not take any drugs known to interfere with CYP3A enzymes (41) for at least 2 weeks before surgery. The CYP3A concentrations vary considerably among different locations in the small intestine (42). To avoid this type of variation, only duodenal samples taken from equivalent locations were included in the study. Twelve nmol of tacrolimus was added to 1 ml human small intestinal microsomes (1.5 g/liter) in 0.1 M phosphate buffer (pH 7.4) and 0.5 ml NADPH generating system. After incubation at 37°C for 20 min, the reaction was stopped by adding 500 μl acetonitrile. Distribution statistics were calculated using the univariate procedure, and the 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).

Fig. 3. LC/MS analysis of tacrolimus and its metabolites after incubation with human or pig small intestinal microsomes.

Three representative ion chromatograms of an analytical run are shown. Incubations of 12 nmol tacrolimus with pig small intestine microsomes (A), human liver microsomes (B), and human small intestinal microsomes (C) for 20 min as described in Materials and Methods. Arrows indicate injection.
Identification of the CYP Enzymes Responsible for Tacrolimus Metabolism. Chemical Inhibition of Tacrolimus Metabolism in Human Small Intestine Microsomes. Preincubation of human small intestinal microsomes with 250 μM troleandomycin reduced generation of 13-O-demethyl tacrolimus by 85% (fig. 4). When 250 μM (final concentration) erythromycin were added to the reaction mixture, the production of 13-O-demethyl tacrolimus was also significantly inhibited (>80%).

Fig. 4. Chemical inhibition of tacrolimus demethylation by troleandomycin (TAO) in human small intestine microsomes.

Inhibition of 13-O-demethyl tacrolimus formation following a 20-min preincubation period with different concentrations of troleandomycin. One ml small intestinal microsomal protein (1.5 g/liter), 50 μl NADPH generating system, and various concentrations of troleandomycin were incubated at 37°C for 20 min. Then, 20 μM tacrolimus, 450 μl NADPH generating system, and 900 μl of 0.1 M phosphate buffer (pH 7.4) were added and incubated at 37°C for 15 min. All values are means ± SDs (N = 4).

Immuno inhibition of Tacrolimus Metabolism. To identify the CYP enzymes responsible for the intestinal metabolism of tacrolimus, microsomes that were isolated from human enterocytes were preincubated with antibodies against various CYP enzymes. Only anti-CYP3A inhibited the formation of 13-O-demethyl tacrolimus, whereas anti-CYP2E1, anti-CYP2C, and anti-CYP1A had no inhibitory effects. At a concentration of 10 mg CYP3A antibody/mg protein, formation of 13-O-demethyl tacrolimus was reduced by 75% (fig. 5).

Fig. 5. Immuno inhibition of tacrolimus metabolism in human small intestine microsomes.

Inhibition of the 13-O-demethyl tacrolimus formation in the presence of different CYP3A antibody concentrations. Anti-CYP3A4 was preincubated for 15 min with human small intestine microsomes and an NADPH generating system. Then, 8.3 μM tacrolimus was added and incubated for 20 min. To the negative control samples, preimmune rabbit IgG was added. Data are presented as means ± SEs (N = 4). O, preimmune immunoglobulin G; ●, anti-CYP3A immunoglobulin G.

Induction of Tacrolimus Metabolism in the Rat Small Intestine. In comparison with microsomes from untreated rats, the specific CYP3A inducer dexamethasone increased tacrolimus metabolite formation. Representative HPLC/UV chromatograms after incubation of tacrolimus with microsomes isolated from rats that had been treated with various CYP inducers are shown in fig. 6. The metabolite patterns calculated as the ratios between single metabolites, including tacrolimus and its demethylated, didemethylated, and hydroxylated metabolites after HPLC/MS analysis, were not significantly changed. The other CYP enzyme inducers included in the study failed to alter the metabolism of tacrolimus. It was concluded from the following results that CYP3A enzymes are responsible for O-demethylation of tacrolimus in the small intestine: formation of 13-O-demethyl tacrolimus formation was inhibited by the specific, competitive CYP3A inhibitor erythromycin; the specific, noncompetitive CYP3A inhibitor troleandomycin; and specific CYP3A antibodies. The formation rate of 13-O-demethyl tacrolimus was only increased after pretreatment of rats with the CYP3A inducer dexamethasone.

CYP3A in Small Intestine of Rat, Humans, and Pig. The drug interaction studies required large quantities of small intestinal microsomes, which were not available from human or rat small intestine. Therefore, pig small intestinal microsomes were used. The presence of enzymes of the CYP3A subfamily was assessed by immunoblot analysis (fig. 7). The human CYP3A antibody recognized the CYP3A proteins in human liver and small intestine microsomes, as well as in rat liver and pig small intestine microsomes. This cross-reactivity of the antibody suggests at least partial homology between human CYP3A and the equivalent CYP in pig intestinal microsomes. The tacrolimus metabolite pattern as checked by HPLC/MS analysis was equal to that generated by human intestinal microsomes (fig. 3). The $K_M$'s and $V_{max}$'s for the 13-O-demethylation of tacrolimus were equal in human liver and pig small intestine microsomes: human liver microsomes—$K_M = 6.9 ± 1.2$ μM, $V_{max} = 66 ± 8.6$ pmol...
FIG. 6. Metabolism of tacrolimus after pretreatment of rats with CYP inducers.

Rat small intestinal microsomes from rats (1.5 g/liter microsomal protein) were incubated for 20 min at 37°C with 10 µM tacrolimus and an NADPH generating system. (A) Incubation with microsomes from untreated rats; (B) from dexamethasone-treated rats; (C) from phenobarbital-treated rats; (D) 3-methylcholanthrene-treated rats; and (E) negative control [0.1 M phosphate buffer (pH 7.4) instead of a NADPH generating system]. Arrows indicate the relative retention time of 13-O-demethyl tacrolimus (peak 1, tR = 12 min) and tacrolimus (peak 2, tR = 26 min). Experiments were repeated 5 times, and representative chromatograms after HPLC/UV analysis are shown.

FIG. 7. Detection of CYP3A enzymes in rat liver, human small intestine and liver, and pig small intestine using immunoblotting.

Microsomes prepared from the villus-tip enterocytes of the proximal intestine, as well as from the liver of rat, human, and pig were subjected to electrophoresis in a polyacrylamide gel containing sodium dodecyl sulfate. Separated proteins were transferred electrophoretically to polyvinylidene difluoride sheets, and the resulting blots were stained using a CYP3A antibody. Lane 1, molecular weight marker; lane 2, rat liver microsomes (3 µg protein); lanes 3 and 4, human liver microsomes (20 µg protein); lanes 5 and 6, human small intestine microsomes (20 µg protein); and lanes 7 and 8, pig small intestine microsomes (lane 7, 50 µg protein; lane 8, 30 µg protein).

Product ∙ min⁻¹ ∙ mg protein (mean ± SD, N = 5); and pig small intestine microsomes—Km = 5.6 ± 2.3 µM, Vmax = 60 ± 26 pmol ∙ min⁻¹ ∙ mg (N = 24).

Immunoblot analysis with antibodies against CYP1A and CYP2C gave positive results with liver and small intestinal microsomes of humans, rat, and pig. Although CYP2E1 was detectable in liver microsomes of all species included in the study, it was only detectable in the small intestinal microsomes of rats.

As in human small intestinal microsomes, troleandomycin was an effective inhibitor of tacrolimus metabolism in rat and pig small intestinal microsomes, decreasing 13-O-demethyl tacrolimus formation at concentrations > 0.1 µM. At a troleandomycin concentration of 250 µM tacrolimus metabolism was reduced by 85%.

In Vitro Drug Interactions with the Metabolism of Tacrolimus in Pig Small Intestine Microsomes. Of the 32 xenobiotics tested, 17 were found to have no effect on the in vitro metabolism of tacrolimus by pig small intestine microsomes: acetyl salicylic acid, amphotericin B, α-naphthoflavone, captopril, cimetidine, diclofenac, diethyldithiocarbamate, disulfiram, lidocaine, naringinine, phenytoin, paracetamol, propranolol, quinidine, ranitidine, sulfaphenazol, and trimethoprim.

For a list of drugs that inhibited tacrolimus metabolism, the type of inhibition, and Km please refer to table 1.

Interindividual Variability. The interindividual variability of tacrolimus metabolism was evaluated in samples collected from the duodenum of 14 patients (10 males and 4 females) (table 2).

The formation rate of 13-O-demethyl tacrolimus were homogeneously distributed and covered a range from 24.1 ± 6.9 to 110.4 ± 7.3 pmol/min/mg (mean ± SD, N = 3), indicating interindividual variation by a factor of 5. The mean 13-O-demethyl tacrolimus formation was significantly higher in microsomes from female than from male duodenum samples, with p < 0.02 (Wilcoxon test).

Discussion

The results of this study demonstrate that the metabolism of tacrolimus, a known CYP3A substrate in the human liver (21–23), is metabolized by the same enzyme CYP subfamily in the small intes-
The tacrolimus metabolism patterns generated in vitro by human small intestinal microsomes equal to those generated by human liver microsomes. The drugs that inhibit tacrolimus metabolism in the liver are inhibitors of small intestinal metabolism as well. Small intestinal metabolism interindividually varies about five times, and duodenum samples from female patients have a significantly higher CYP3A activity than samples from male patients.

The analytical HPLC/UV method used in this study allowed the specific detection of 13-O-demethyl and 13,15-O-didemethyl tacrolimus (34). All other metabolites eluted in peaks that represented a mixture of several different metabolites. HPLC/MS allowed quantification of metabolites with different m/z and similar retention times. However, this method was unable to differentiate between metabolites with equal HPLC retention times and the same m/z, such as 13-O-, 15-O-, and 31-O-demethyl tacrolimus. It was possible to quantify metabolites, the structures of which have not yet been completely identified, such as dihydroxy tacrolimus (12). In contrast to metabolites measured by HPLC/UV, it was not possible to give the exact structure of the metabolites after measurement by HPLC/MS. Because clinical studies have shown that demethylated metabolites are the major metabolites in blood of patients (13), the present study mainly focused on these metabolites.

In most drug interaction studies to date, it has been assumed that interactions at the CYP monooxygenase level leading to significant changes of pharmacokinetic parameters are exclusively located in the liver. Several studies evaluating the pharmacokinetic drug interactions of the CYP3A substrate cyclosporine have provided evidence that CYP3A inhibitors increase and CYP3A inductors reduce absorption and blood concentrations of cyclosporine (17, 19, 43). Cyclosporine metabolites have been detected after incubation with small intestinal microsomes (18, 44) and in the Ussing chamber (45). Kolars et al. (46) demonstrated significant cyclosporine intestinal metabolism in anhepatic patients undergoing liver transplantation. Because in rat cyclosporine was metabolized in the small intestine and the metabolites were mainly found in the lumen, it was hypothesized that small intestinal metabolism might be a determinant of oral bioavailability (18). The results of our study prove that tacrolimus is a CYP3A substrate in the small intestine. It can be speculated that the results of the cyclosporine studies discussed herein apply for tacrolimus as well, and that metabolism of tacrolimus in the small intestine might be the reason for its low and highly variable bioavailability.

Interaction studies of xenobiotics with the small intestinal metabolism of tacrolimus required the development of a readily and easily available model, because rat and human small intestinal microsomes...

### TABLE 1

Table 1: $K_i$ values and mechanism of inhibition of drugs that interacted with the in vitro 13-O-demethyl tacrolimus formation by human liver microsomes and pig small intestinal microsomes. These $K_i$ are given as mean ± SD ($N = 5$).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Pig Small Intestine</th>
<th>Competitive Inhibitor</th>
<th>Human Liver</th>
<th>Competitive Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (nM/lit)</td>
<td></td>
<td>$K_i$ (nM/lit)</td>
<td></td>
</tr>
<tr>
<td>Bromocryptine</td>
<td>28 ± 2.4</td>
<td>-</td>
<td>7 ± 2.7</td>
<td>-</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>164 ± 9.6</td>
<td>-</td>
<td>121 ± 7.5</td>
<td>-</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>62 ± 7.4</td>
<td>+</td>
<td>37 ± 8.4</td>
<td>+</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>95 ± 5.7</td>
<td>+</td>
<td>23 ± 6.0</td>
<td>+</td>
</tr>
<tr>
<td>Ergotamine</td>
<td>100 ± 7.6</td>
<td>-</td>
<td>14 ± 4.2</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>43 ± 6.7</td>
<td>+</td>
<td>16 ± 4.8</td>
<td>+</td>
</tr>
<tr>
<td>Ethynyl estradiol</td>
<td>249 ± 8.7</td>
<td>-</td>
<td>34 ± 6.0</td>
<td>-</td>
</tr>
<tr>
<td>Josamycin</td>
<td>50 ± 4.1</td>
<td>-</td>
<td>21 ± 4.2</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2 ± 0.9</td>
<td>-</td>
<td>8 ± 4.0</td>
<td>-</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>6 ± 1.4</td>
<td>+</td>
<td>12 ± 5.3</td>
<td>+</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>65 ± 3.5</td>
<td>+</td>
<td>79 ± 9.0</td>
<td>+</td>
</tr>
<tr>
<td>Progesterone</td>
<td>5 ± 1.7</td>
<td>-</td>
<td>8 ± 4.0</td>
<td>-</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>34 ± 5.2</td>
<td>+</td>
<td>83 ± 7.3</td>
<td>+</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>2 ± 1.3</td>
<td>-</td>
<td>51 ± 5.9</td>
<td>-</td>
</tr>
<tr>
<td>Verapamil</td>
<td>63 ± 6.4</td>
<td>-</td>
<td>82 ± 7.3</td>
<td>-</td>
</tr>
</tbody>
</table>

### TABLE 2

Table 2: Interindividual variability of the metabolism of tacrolimus by human small intestinal microsomes.

<table>
<thead>
<tr>
<th>Patients</th>
<th>13-O-demethyl tacrolimus Formation Rate (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89.1 ± 11.6</td>
</tr>
<tr>
<td>2</td>
<td>110.2 ± 7.3</td>
</tr>
<tr>
<td>3</td>
<td>51.7 ± 5.1</td>
</tr>
<tr>
<td>4</td>
<td>102.4 ± 4.1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>88.4 ± 25.9</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>24.1 ± 6.9</td>
</tr>
<tr>
<td>6</td>
<td>28.1 ± 8.4</td>
</tr>
<tr>
<td>7</td>
<td>24.7 ± 3.2</td>
</tr>
<tr>
<td>8</td>
<td>52.7 ± 5.7</td>
</tr>
<tr>
<td>9</td>
<td>33.4 ± 3.8</td>
</tr>
<tr>
<td>10</td>
<td>29.3 ± 5.4</td>
</tr>
<tr>
<td>11</td>
<td>51.5 ± 12.8</td>
</tr>
<tr>
<td>12</td>
<td>52.3 ± 5.9</td>
</tr>
<tr>
<td>13</td>
<td>33.2 ± 2.2</td>
</tr>
<tr>
<td>14</td>
<td>76.6 ± 4.1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>54.2 ± 29.2</td>
</tr>
</tbody>
</table>
can usually be obtained only in small quantities. It has been shown that the metabolism of the CYP3A substrate midazolam is different in humans and in the pig. Although human metabolism of midazolam results mainly in generation of 1'-hydroxy midazolam (47), 4-hydroxy midazolam was the major metabolite in pigs (48). Therefore, it was essential to prove that no such differences between human and pig small intestinal microsomal metabolism existed for tacrolimus. Analysis of the tacrolimus metabolite pattern after metabolism by pig small intestinal microsomes in comparison with that after metabolism by human small intestinal microsomes, as well as similar $K_M$ and $V_{max}$ for the O-demethylation of tacrolimus by both microsomal preparations, suggested that pig microsomes represent a valid model to study tacrolimus small intestinal metabolism. However, the $K_s$'s of several drugs—such as bromocryazine, dexamethasone, ergotamine, erythromycin, and josamycin—were more than twice higher, compared with human liver microsomes when pig small intestine microsomes were used. On the other hand, the $K_s$'s of troledonmycin, nifedipine, and ketokonazole were lower in pig small intestine than in human liver. Whether these differences have to be either attributed to a species-specific or an organ-specific difference could not be decided from the data. Because the same drugs were found to inhibit tacrolimus metabolism in the pig small intestine as in the human liver, it can be concluded that these inhibitors inhibit tacrolimus metabolism in the human small intestine as well.

All drugs found to interact with the small intestinal metabolism of tacrolimus are well known CYP3A substrates and/or inhibitors (41, 49). Some known CYP3A substrates, such as lidocaine (50), did not interact with the small intestinal metabolism of tacrolimus that might be explained by the lower affinity of these substrates (lidocaine: $K_M = 119 \mu M$ to CYP3A enzymes, compared with tacrolimus ($K_M = 6.7 \mu M$) (24). Several xenobiotics that were included in the study are known as specific inhibitors of CYP enzymes different from CYP3A, such as $\alpha$-naphthoflavone (CYP1A1) (37), sulfaphenazole (CYP2C) (50, 51), quinidine and propanol (CYP2D6) (52), and diethylthiocarbamate and disulfiram (CYP2E1) (38, 50). None of these were effective inhibitors of the metabolism of tacrolimus by pig small intestinal microsomes, excluding the involvement of the respective CYP enzymes.

The results of this in vitro study have to be transferred to the in vivo situation with caution. In vitro, the xenobiotics are readily available for metabolism by CYP enzymes, whereas in vivo other parameters such as intestinal transport, drug release, composition of the intestinal fluid, intestinal pH, protein binding and blood and lymph flow may influence the availability of the drug for CYP enzymes. The relevance of the results of this study remains to be evaluated in clinical studies.

As previously discussed, several clinical studies provided evidence for a significant contribution of small intestinal metabolism to the bioavailability after oral application. The homogeneously distributed and highly variable activity of CYP3A enzymes in human proximal small intestine shown in the present study may serve as an explanation for the variable bioavailability of several CYP3A substrates. The CYP3A distribution pattern in small intestinal microsomes of 14 patients is comparable with that described by Schellens et al. (53) and Wrighton et al. (54) for CYP3A enzymes in the liver and the interpatient heterogeneity in expression of CYP3A4 in the small intestine described by Lown et al. (55).

The present study showed an approximately 60% higher CYP3A activity in female duodenal samples than in male samples. All samples were taken from equal locations of the small intestine, and samples from patients taking drugs influencing CYP3A activities were excluded. Such a gender-dependent difference of CYP3A activity has not yet been reported before. However, Hunt et al. (56) found a 24% higher activity of CYP3A enzymes in livers of female patients compared with male patients. Whether or not the gender-dependent activity of CYP3A in the small intestine found in the present study is a relevant result requires further assessment, including a larger number of samples.

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