EFFECT OF CYP3A5 POLYMORPHISM ON TACROLIMUS METABOLIC CLEARANCE IN VITRO

Yang Dai, Mary F. Hebert, Nina Isoherranen, Connie L. Davis, Christopher Marsh,1 Danny D. Shen, and Kenneth E. Thummel

Department of Pharmaceutics (Y.D., N.I., D.D.S., K.E.T.), Department of Pharmacy (M.F.H., D.D.S.), Department of Medicine, Division of Nephrology (C.L.D.), and Department of Surgery (C.M.), University of Washington, Seattle, Washington

Received November 30, 2005; accepted February 14, 2006

ABSTRACT:

Previous investigations of solid organ transplant patients treated with tacrolimus showed that individuals carrying a CYP3A5*1 allele have lower dose-adjusted trough blood concentrations compared with homozygous CYP3A5*3 individuals. The objective of this investigation was to quantify the contribution of CYP3A5 to the hepatic and renal metabolic clearance of tacrolimus. Four primary tacrolimus metabolites, 13-O-desmethyl tacrolimus (13-DMT) (major), 15-O-desmethyl tacrolimus, 31-O-desmethyl tacrolimus (31-DMT), and 12-hydroxy tacrolimus (12-HT), were generated by human liver microsomes and heterologously expressed CYP3A4 and CYP3A5. The unbound tacrolimus concentration was low (4–15%) under all incubation conditions. For CYP3A4 and CYP3A5, V_{\text{max}}^\text{u} was 8.0 and 17.0 nmol/min/mmol enzyme and K_{\text{m,u}} was 0.21 and 0.21 μM, respectively. The intrinsic clearance of CYP3A5 was twice that of CYP3A4. The formation rates of 13-DMT, 31-DMT, and 12-HT were ≈1.7-fold higher, on average, in human liver microsomes with a CYP3A5*1/3 genotype compared with those with a homozygous CYP3A5*3/3 genotype. Tacrolimus disappearance clearances were 15.9 ± 9.8 ml/min/mg protein and 6.1 ± 3.6 ml/min/mg protein, respectively, for the two genotypes. In vitro to in vivo scaling using both liver microsomes and recombinant enzymes yielded higher predicted in vivo tacrolimus clearances for patients with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. In addition, formation of 13-DMT was 13.5-fold higher in human kidney microsomes with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. These data suggest that CYP3A5 contributes significantly to the metabolic clearance of tacrolimus in the liver and kidney.

Financial support for this work was provided in part by grants from the National institutes of Health: GM63666, GM32165, and ES07033.

1 Current affiliation: Center for Organ and Cell Transplantation, Scripps Green Hospital, La Jolla, California.

Page 836

ABSTRACT: Previous investigations of solid organ transplant patients treated with tacrolimus showed that individuals carrying a CYP3A5*1 allele have lower dose-adjusted trough blood concentrations compared with homozygous CYP3A5*3 individuals. The objective of this investigation was to quantify the contribution of CYP3A5 to the hepatic and renal metabolic clearance of tacrolimus. Four primary tacrolimus metabolites, 13-O-desmethyl tacrolimus (13-DMT) (major), 15-O-desmethyl tacrolimus, 31-O-desmethyl tacrolimus (31-DMT), and 12-hydroxy tacrolimus (12-HT), were generated by human liver microsomes and heterologously expressed CYP3A4 and CYP3A5. The unbound tacrolimus concentration was low (4–15%) under all incubation conditions. For CYP3A4 and CYP3A5, V_{\text{max}}^\text{u} was 8.0 and 17.0 nmol/min/mmol enzyme and K_{\text{m,u}} was 0.21 and 0.21 μM, respectively. The intrinsic clearance of CYP3A5 was twice that of CYP3A4. The formation rates of 13-DMT, 31-DMT, and 12-HT were ≈1.7-fold higher, on average, in human liver microsomes with a CYP3A5*1/3 genotype compared with those with a homozygous CYP3A5*3/3 genotype. Tacrolimus disappearance clearances were 15.9 ± 9.8 ml/min/mg protein and 6.1 ± 3.6 ml/min/mg protein, respectively, for the two genotypes. In vitro to in vivo scaling using both liver microsomes and recombinant enzymes yielded higher predicted in vivo tacrolimus clearances for patients with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. In addition, formation of 13-DMT was 13.5-fold higher in human kidney microsomes with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. These data suggest that CYP3A5 contributes significantly to the metabolic clearance of tacrolimus in the liver and kidney.

Financial support for this work was provided in part by grants from the National institutes of Health: GM63666, GM32165, and ES07033.

1 Current affiliation: Center for Organ and Cell Transplantation, Scripps Green Hospital, La Jolla, California.

Page 836

ABSTRACT: Previous investigations of solid organ transplant patients treated with tacrolimus showed that individuals carrying a CYP3A5*1 allele have lower dose-adjusted trough blood concentrations compared with homozygous CYP3A5*3 individuals. The objective of this investigation was to quantify the contribution of CYP3A5 to the hepatic and renal metabolic clearance of tacrolimus. Four primary tacrolimus metabolites, 13-O-desmethyl tacrolimus (13-DMT) (major), 15-O-desmethyl tacrolimus, 31-O-desmethyl tacrolimus (31-DMT), and 12-hydroxy tacrolimus (12-HT), were generated by human liver microsomes and heterologously expressed CYP3A4 and CYP3A5. The unbound tacrolimus concentration was low (4–15%) under all incubation conditions. For CYP3A4 and CYP3A5, V_{\text{max}}^\text{u} was 8.0 and 17.0 nmol/min/mmol enzyme and K_{\text{m,u}} was 0.21 and 0.21 μM, respectively. The intrinsic clearance of CYP3A5 was twice that of CYP3A4. The formation rates of 13-DMT, 31-DMT, and 12-HT were ≈1.7-fold higher, on average, in human liver microsomes with a CYP3A5*1/3 genotype compared with those with a homozygous CYP3A5*3/3 genotype. Tacrolimus disappearance clearances were 15.9 ± 9.8 ml/min/mg protein and 6.1 ± 3.6 ml/min/mg protein, respectively, for the two genotypes. In vitro to in vivo scaling using both liver microsomes and recombinant enzymes yielded higher predicted in vivo tacrolimus clearances for patients with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. In addition, formation of 13-DMT was 13.5-fold higher in human kidney microsomes with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. These data suggest that CYP3A5 contributes significantly to the metabolic clearance of tacrolimus in the liver and kidney.

Financial support for this work was provided in part by grants from the National institutes of Health: GM63666, GM32165, and ES07033.

1 Current affiliation: Center for Organ and Cell Transplantation, Scripps Green Hospital, La Jolla, California.

Page 836

ABSTRACT: Previous investigations of solid organ transplant patients treated with tacrolimus showed that individuals carrying a CYP3A5*1 allele have lower dose-adjusted trough blood concentrations compared with homozygous CYP3A5*3 individuals. The objective of this investigation was to quantify the contribution of CYP3A5 to the hepatic and renal metabolic clearance of tacrolimus. Four primary tacrolimus metabolites, 13-O-desmethyl tacrolimus (13-DMT) (major), 15-O-desmethyl tacrolimus, 31-O-desmethyl tacrolimus (31-DMT), and 12-hydroxy tacrolimus (12-HT), were generated by human liver microsomes and heterologously expressed CYP3A4 and CYP3A5. The unbound tacrolimus concentration was low (4–15%) under all incubation conditions. For CYP3A4 and CYP3A5, V_{\text{max}}^\text{u} was 8.0 and 17.0 nmol/min/mmol enzyme and K_{\text{m,u}} was 0.21 and 0.21 μM, respectively. The intrinsic clearance of CYP3A5 was twice that of CYP3A4. The formation rates of 13-DMT, 31-DMT, and 12-HT were ≈1.7-fold higher, on average, in human liver microsomes with a CYP3A5*1/3 genotype compared with those with a homozygous CYP3A5*3/3 genotype. Tacrolimus disappearance clearances were 15.9 ± 9.8 ml/min/mg protein and 6.1 ± 3.6 ml/min/mg protein, respectively, for the two genotypes. In vitro to in vivo scaling using both liver microsomes and recombinant enzymes yielded higher predicted in vivo tacrolimus clearances for patients with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. In addition, formation of 13-DMT was 13.5-fold higher in human kidney microsomes with a CYP3A5*1/3 genotype compared with those with a CYP3A5*3/3 genotype. These data suggest that CYP3A5 contributes significantly to the metabolic clearance of tacrolimus in the liver and kidney.

Financial support for this work was provided in part by grants from the National institutes of Health: GM63666, GM32165, and ES07033.

1 Current affiliation: Center for Organ and Cell Transplantation, Scripps Green Hospital, La Jolla, California.

Page 836
copies of a variant allele (CYP3A5*3, *6, or *7) accounts for a markedly reduced level of CYP3A5 protein expression and function in approximately 85% of whites, 65% of Chinese, and 55% of African Americans (Lamba et al., 2002). Moreover, inheritance of the CYP3A5*1 allele is strongly correlated with enhanced CYP3A5-dependent metabolism in the liver in vitro (Kuehl et al., 2001; Lin et al., 2002; Huang et al., 2004).

Several recent studies of heart, lung, and kidney transplantation patients document a significant association between the CYP3A5 polymorphism and tacrolimus dose-adjusted trough blood levels (Hesselink et al., 2003; Thervet et al., 2003; Zheng et al., 2003; Haufroid et al., 2004). Interestingly, this trend is not as obvious in cyclosporine-treated patients (Hesselink et al., 2003; Haufroid et al., 2004; Kreutz et al., 2004), even though cyclosporine is also a CYP3A5 substrate (Aoyama et al., 1989; Dai et al., 2004). This could be due to differences in the relative contribution of each enzyme toward cyclosporine and tacrolimus metabolism. CYP3A4 catalyzes the formation of all three major primary cyclosporine metabolites (AM1, AM9, and AM4N), whereas only AM9 is produced to any significant degree by CYP3A5. In addition, cyclosporine intrinsic clearance (CL\textsubscript{int}), calculated from total metabolite formation, is approximately 2.3-fold higher for CYP3A4 than for CYP3A5 (Dai et al., 2004).

For tacrolimus, Bader et al. (2000) showed that CYP3A5 generates both desmethyl and hydroxyl metabolites and that CYP3A4 generated the desmethyl but not the hydroxyl metabolite. Thus, it is possible that CYP3A5 contributes more significantly to the systemic clearance of tacrolimus than does CYP3A4 in patients carrying the CYP3A5*1 allele. With respect to the renal disposition of tacrolimus, patients with a CYP3A5*1/*3 genotype may exhibit higher rates of renal tacrolimus metabolism than those lacking the *1 allele, which would result in differences in intrarenal accumulation of tacrolimus, independent of systemic blood concentration.

Based on this background, we hypothesized that CYP3A5 contributes more significantly to the metabolic clearance of tacrolimus than does CYP3A4 and that inheritance of CYP3A5*1 allele in patients treated with tacrolimus has a protective role in the development of drug-related nephrotoxicity. The purpose of this study was: 1) to determine the unbound Michaelis constant (K\textsubscript{m,u}) and CL\textsubscript{int,u} for tacrolimus metabolism by CYP3A4 and CYP3A5, and 2) to assess the relative contribution of CYP3A5 to total tacrolimus metabolism by human liver and kidney tissues.

**Materials and Methods**

**Materials.** Tacrolimus was kindly provided by Fujisawa USA Inc. (now Astellas Pharma US, Inc., Deerfield, IL). Ascomycin and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Methanol, acetonitrile, and ethyl acetate were purchased from Fisher Scientific (Santa Clara, CA). Heterologous baculovirus-insect cell-expressed human CYP3A4/P450 reductase (rCYP3A4), CYP3A5/P450 reductase (rCYP3A5), CYP3A4/P450 reductase + cytochrome b\textsubscript{5} (rCYP3A4+b\textsubscript{5}), CYP3A5/P450 reductase + cytochrome b\textsubscript{5} (rCYP3A5+b\textsubscript{5}),

![Fig. 1. Metabolic pathway of tacrolimus. The sites of metabolism are numbered in tacrolimus structure and are indicated by an arrow in the corresponding metabolite structures. Note: 13-DMT and 12-HT undergo furan ring rearrangement.](image-url)
and CYP3A7 + P450 reductase + cytochrome \( b_6 \) (rCYP3A7 + \( b_6 \)) Supersomes were purchased from BD Gentest (Woburn, MA). Human cytochrome \( b_6 \) was purchased from PanVera (Carlsbad, CA).

**Preparation of Human Liver and Kidney Microsomes.** The collection and use of human tissue for research was approved by the University of Washington Human Subjects Review Board. Liver tissue from white donors was obtained from the University of Washington, School of Pharmacy Human Tissue Bank (Seattle, WA). Kidney tissue was obtained from the National Disease Research Interchange (Philadelphia, PA). Of the 60 available livers, 10 from a subset of CYP3A5*1/*3 donors were selected randomly for microsome preparation. Liver tissue from another 10 donors with a CYP3A5*5*/5*3 genotype were selected and microsomes prepared to obtain 10 paired CYP3A5*1*/3*3 and CYP3A5*3*/5*3 preparations with the same CYP3A4 immunocent for each pair. Of the 21 kidneys available, all four with a CYP3A5*1*/1*3 genotype (two from white donors, one from a Hispanic donor, and one from a donor with unknown ethnicity) were selected for microsomal preparation. Microsomes were prepared from another four kidneys (all from white donors) selected randomly from the CYP3A5*5*/3*3 donor pool. The procedures used for microsome isolation have been published previously (Paine et al., 1997; Lin et al., 2002; Givens et al., 2003; Dai et al., 2004). Protein concentrations were determined using bovine serum albumin as the reference standard.

**Isolation and Mass Spectrometric Analysis of Tacrolimus Metabolites.** Tacrolimus metabolites were generated and isolated as described previously (Iwasaki et al., 1993), with some modifications. Tacrolimus (100 \( \mu \)M) was incubated with pooled human liver microsomes (3 mg/ml) supplemented with 1 mM NADPH for 45 min in a volume of 30 ml. The reaction mixture was extracted twice with 30 ml of ethyl acetate, and the organic layer was collected and evaporated to dryness under a stream of nitrogen at 30°C. The residue was reconstituted in 90 \( \mu \)l of acetonitrile and injected onto a semipreparative column (Ultremex 5 C18, 250 \( \times \) 10 mm; Phenomenex, Torrance, CA) heated to 50°C. The mobile phases were A, \( \mathrm{H}_2 \mathrm{O} \); and B, acetonitrile. The gradient was 37% B at 0 min, 62% B at 30 min, and 100% B at 30.5 min, and the flow rate was 4.5 ml/min. Fractions were collected manually at a 1-min interval. The volume of each fraction was reduced to about one-third under a stream of nitrogen to remove acetonitrile and transferred to a lyophilizer to remove water and residual organic solvent. Fractions 1 to 4 (Fig. 2A) contained the major NADPH-dependent primary tacrolimus metabolites identified by pilot small-scale (1-ml) incubations with and without the addition of NADPH. Each fraction was reconstituted in 60% acetonitrile (80 \( \mu \)l) and reseparated with an analytical C18 column heated to 50°C. The mobile phases were (A, 0.4% acetic acid/\( \mathrm{H}_2 \mathrm{O} \); and B, acetonitrile). Different isocratic conditions were used for fractions 1 and 2 (50% B), and fractions 3 and 4 (55% B). The flow rate was 1 ml/min. Peaks were collected and the solvent was removed as described above.

**Identification of Metabolites.** The isolated metabolites were identified based on their LC/MS-MS mass fragmentation patterns. Liquid chromatography was performed on a Shimadzu LC-10AD solvent delivery system equipped with a Shimadzu SIL-10ADVP autoinjector (Shimadzu Scientific Instruments, Inc., Columbia, MD), a Develosil ODS-HG-5, 150 \( \times \) 2.0 mm, 5-\( \mu \)m analytical column (Nomura Chemical Co., Ltd., Seto, Japan), and a SecurityGuard column (Phenomenex), with both columns heated to 50°C. Two mobile phases (A, 0.05% TFA and 0.25 \( \mu \)M sodium acetate in \( \mathrm{H}_2 \mathrm{O} \); B, acetonitrile) and a stepwise linear gradient procedure (45% B at 0 min; 65% B at 16.5 min; 100% B between 16.6 and 19.0 min) was used to separate the tacrolimus metabolites. Mass spectrometry was performed on a Micromass Quadruple II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK). The positive electrospray (ESP+) mode was used. High-performance liquid chromatography eluate was introduced into a stainless steel capillary sprayer held at 3.5 kV through 50-\( \mu \)m silica tubing with a desolvation temperature of 400°C. Each fragment was analyzed under scan and SIR modes. When the SIR mode was
used, m/z values were selected based on results from the scan. The fragmentation patterns of the sodium adducts of tacrolimus and metabolites were obtained using the daughter ion function. The cone voltage was 50 eV and the collision energy was adjusted to get optimal fragmentation patterns (40–50 eV).

Once identified, concentrations of the metabolite stock solutions (prepared from dried purified metabolites as described above) were assigned by UV absorbance using a standard curve of tacrolimus, assuming a common extinction coefficient at 214 nm. This approach has been used for both tacrolimus (Lampen et al., 1995) and cyclosporine (Wallenmaq et al., 1989; Brooks et al., 1993) when no authentic metabolite standards were available and gravimetric measurement was impossible because of the small amount of metabolites isolated. An analytical column (Microsorb-MV C18 250 × 4.6 mm; Varian, Walnut Creek, CA) was used for separation and was heated to 50°C. The mobile phases were A, 0.05% phosphoric acid/H2O and B, acetonitrile. A stepwise linear gradient was used: 37% B at 0 min, 65% B at 35 min and maintained for 5 min, 100% B at 40.5 min and maintained for 5 min. The flow rate was 1 ml/min. Peaks other than the purified tacrolimus metabolites were not observed under UV detection. The purity of the metabolites was assessed by inspection of the LC/MS total ion current for the injected standard and comparison of the observed and published fragmentation patterns using LC/MS-MS.

**LC/MS Quantification of Metabolites.** The same LC/MS conditions described above for metabolite identification were applied for their quantification after incubation of tacrolimus with the different enzyme systems, with slight modification. Solvent A was 0.05% TFA and 0.25 μM potassium acetate (instead of sodium acetate) in H2O. For MS detection, the cone voltage was held at 50 eV. Sodium and potassium adducts were detected for detection limit was 0.01 (instead of sodium acetate) in H2O. For MS detection, the cone voltage was 400 μg/l for liver microsomal incubations. The detection and quantitation limits of tacrolimus were 0.1 and 0.5 μg/l. Intraday and interday variability for the quantitation of tacrolimus was less than 10%.

**Nonspecific Binding of Tacrolimus in Vitro.** The unbound concentration of tacrolimus under the same conditions used for each of the substrate disappearance and metabolite formation experiments was measured by ultrafiltration. For each experimental condition used (enzyme and substrate concentration), kinetic parameters were determined in duplicate, and the average value is reported. Each reaction mixture (1 ml) was prepared in 15-ml centrifuge tubes and mixed thoroughly. A portion of the solution (50 μl) was removed to measure the initial concentration in the solution ([S]0). The remaining mixture was then transferred to the retainer chamber of a Centrifree ultrafiltration kit (Millipore Corp., Bedford, MA). At the protein concentration used in the experiment, adsorption to the retainer chamber surface and the membrane was negligible. Fifty microliters of BSA solution (10 mg/ml) were put in the acceptor chamber to minimize adsorptive loss to the surface. Each reaction mixture was centrifuged twice at 50gf for 1 and 1.5 min at 37°C. The first ultrafiltrate (~100 μl) was discarded. The second ultrafiltrate (60–100 μl) was collected for measurement of the unbound drug concentration ([S]u).

Tacrolimus bound extensively to the incubation tubes as well as microsomes. As a consequence, the nonspecific binding of tacrolimus was described as follows:

\[ f_u = \frac{[S]_u}{[S]_u} \]  \hspace{1cm} (1)

\[ f_u = \frac{[S]_u}{[S]_u} \] \hspace{1cm} (2)

\[ f_{u,app} = \frac{[S]_u}{[S]_u} \times f_u \] \hspace{1cm} (3)

where \([S]_u\) is the nominal initial concentration of the reaction mixture; \([S]_u\) is the total drug concentration in the aqueous mixture after addition to an incubation tube (concentration for the total amount of drug minus the amount of drug bound to the tube); \([S]_u\) is the unbound drug concentration in the aqueous mixture; \(f_u\) is the fraction of total amount of drug added to the tube that remained in the aqueous environment; \(f_u\) is the fraction of unbound drug in the aqueous mixture (relative to \([S]_u\)); and \(f_{u,app}\) is the apparent unbound drug concentration in the mixture that was used to correct the concentration-dependent metabolite formation or substrate disappearance data.

For heterologously expressed enzyme incubations, the fraction \(f_u\) increased with increasing tacrolimus concentration, ranging from 10 to 50% over a tacrolimus concentration range of 0.02 to 10 μM. In contrast, \(f_u\) was relatively constant with increasing tacrolimus concentration, ranging from 20 to 30% over the same tacrolimus concentration range. As a result, \(f_{u,app}\) increased with increasing tacrolimus concentration. The ranges were 5 to 14% for rCYP3A4, 5 to 16% for rCYP3A5, 3 to 17% for rCYP3A5*1/*3, 5 to 16% for rCYP3A4, and 5 to 15% for rCYP3A5. Under conditions used for liver microsomal incubations (0.2 μM tacrolimus and 0.05 mg/ml microsomes), \(f_u\), \(f_{u,app}\), and \(f_{u,app}\) were 72%, 26%, and 19%, respectively, for CYP3A5*1/*3 microsomes, and 56%, 38% and 21%, respectively, for CYP3A5*1/*3 microsomes. Values for \(f_{u,app}\) were applied to data from subsequent kinetic experiments for estimation of the unbound Michaelis constant (Km,un) and intrinsic clearance (CLint,un).

**Tacrolimus Metabolite Formation Kinetics.** Kinetic Protocol. Incubations of each liver/kidney microsome or recombinant enzyme preparation with each specified concentration of tacrolimus were performed in duplicate in solutions containing 0.1 M potassium phosphate, pH 7.4, and 1 mM EDTA in a shaking water bath maintained at 37°C. Tacrolimus was dissolved in methanol; the final methanol concentration was less than 1%. All reactions were initiated by adding NADPH (final concentration 1 mM) or buffer (as negative control) after 5 min of preincubation and were terminated by adding an equal volume of ice-cold acetonitrile. Conditions that conferred linear metabolite formation with respect to time under different substrate and human liver/kidney microsomal protein concentrations were determined (not shown) and applied to all kinetic experiments described in this study.

For incubations with heterologously expressed enzymes, tacrolimus (0.02–10 μM) was incubated with 10 pmol/ml rCYP3A4 and rCYP3A5...
supplemented with cytochrome b₅ (CYP3A₅b₅ = 1:3) for 2 min (0.02–1 μM) and 1 min (2–10 μM), or 5 pmol/ml CYP3A4 + b₅ and rCYP3A5 + b₅ for 1 min. The final reaction volume was 0.5 ml. The average reaction velocity from replicate incubations was computed for each substrate concentration used. For each recombinant enzyme preparation, full tacrolimus concentration-metabolite formation rate profile experiments were conducted twice on different days. For experiments with human liver microsomes, tacrolimus (0.2 μM) was incubated for 3 min with 0.025 mg of protein from 10 CYP3A₅*1/*₃ donors that were paired one to one with another set of 10 microsomal samples from CYP3A₅*1/*₃ donors, controlling for CYP3A4 immunococontent, as described previously (Daï et al., 2004). The final volume was 0.5 ml. For experiments with human kidney microsomes, 0.2 μM tacrolimus was incubated for 50 min with 0.2 mg of protein from donors with matching CYP3A4 immunococontent, as described above, were used. Tacrolimus (0.2 μM, initial nominal concentration) was added to 0.05 mg/ml microsomal protein; the total incubation volume was 1.6 ml. After preincubation at 37°C for 5 min and addition of NADPH (1 mM final concentration), 200 μl of reaction mixture was removed at 0, 3, 6, and 10 min and mixed with 200 μl of ice-cold acetonitrile.

Reaction products were extracted by adding 20 μl of BSA (5 mg/ml), 100 μl of ascorycin (50 μg/ml), and 3 ml of ethyl acetate. The resulting residue was reconstituted in 200 μl of acetonitrile/H₂O (9:1, v/v) and stored at −20°C. Twenty microliters from each sample was subjected to LC/MS-MS analysis.

**Kinetic Analysis of Substrate Disappearance.** For tacrolimus disappearance experiments using liver microsomes, the apparent substrate disappearance rate constant (Kₑ, time⁻¹) was calculated from the slope of the logarithm of remaining tacrolimus concentration versus incubation time plot after addition of 0.2 μM tacrolimus and NADPH (Obach and Reed-Hagen, 2002). The apparent disappearance clearance (CLₑ,HLM) was calculated from eq. 8:

\[
\text{CLₑ,HLM}} = \frac{Kₑ}{[\text{substrate}]} \quad \text{(8)}
\]

The unbound disappearance clearance (CLₑ,HLM,unbound) was calculated by correcting for the tacrolimus free fraction in the liver microsomal incubation.

\[
\text{CLₑ,HLM,unbound} = \frac{\text{CLₑ,HLM}}{f_u}\times [\text{microsomal protein}] \quad \text{(9)}
\]

Unbound disappearance clearances are reported as the mean (±S.D.) of values from individual livers in each genotype group.

**In Vitro to In Vivo Scaling of Tacrolimus Metabolic Clearance.** The CLₑ,HLM,unbound for each liver microsome preparation were scaled up to estimate the in vivo plasma clearance of tacrolimus (CLₑ,iv) using both well stirred and parallel-tube models. An equation for scaling the intrinsic formation or disappearance clearance was as follows (Naritomi et al., 2001):

\[
\text{CLₑ,iv} = \text{CLₑ,iv} \times \text{(microsomes yield per gram liver)(mg/g)} \times [\text{microsomal protein}] \quad \text{(10)}
\]

where CLₑ,iv was either CLₑ,HLM,unbound or CLₑ,HLM,disapp.unbound. The yield of liver microsomes per gram of liver, the microsomal weight, the liver blood flow, and f_u (splay yield) were fixed to literature values of 48.8 mg/g, 25.7 kg/kg, 20.7 ml/min (Naritomi et al., 2001), and 1% (Nagase et al., 1994; Iwasaki et al., 1996), respectively. Predicted hepatic clearances are reported as mean (±S.D.) of values from individual livers in each genotyped group.

Kinetic parameters estimated from metabolite formation experiments using heterologously expressed enzymes were used to predict a predicted unbound intrinsic liver microsomal clearance (CLₑ,HLM,unbound, predicted) based on the CYP3A4 and CYP3A5 content of the liver microsome preparations used in the study. The predicted unbound formation clearance of liver microsomes for each metabolite (CLₑ,HLM,unbound, predicted) was calculated using eqs. 4 and 5, where CLₑ,HLM,unbound, predicted = Vmax/\text{[S]unbound} . Vmax values were converted from nmol/min/mmol enzyme to nmol/min/mg microsomal protein by use of the CYP3A4 and CYP3A5 immunococontents (pmol of enzyme/mg of microsomal protein) for the corresponding microsomes. CLₑ,HLM,unbound, predicted was the sum of each individual metabolite formation clearance. 15-DMT and 31-DMT formation clearances were not included in the calculation since the kinetic parameters could not be estimated for these metabolites.

\[
\text{CLₑ,HLM,unbound, predicted} = \text{CLₑ,HLM,unbound, predicted, 15-DMT} + \text{CLₑ,HLM,unbound, predicted, 31-DMT} \quad \text{(11)}
\]

Predicted unbound intrinsic formation clearances are reported as mean (±S.D.) of values from individual livers in each genotyped group.

**Spectrophotometric Analysis of Tacrolimus Binding to CYP3A4 and CYP3A5.** UV-visible difference spectra of tacrolimus binding to CYP3A4 and CYP3A5 were obtained using an Aminco DW2 double-beam spectrometer (OLIS, Inc., Bogart, GA). Sample and reference cuvettes each contained a 1-ml incubation mixture consisting of 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, and 200 pmol/ml rCYP3A4 or rCYP3A5. Tacrolimus concentrations were titrated by adding 1 μl of tacrolimus solution (dissolved in methanol) to the sample cuvette to the desired concentration and an equal volume of methanol to the reference cuvette. The difference spectrum was then obtained by scanning from 350 to 500 nm at 37°C. The concentration range was 0 to 19.2 μM for rCYP3A4 and 0 to 19 μM for rCYP3A5. The final methanol
concentration for the highest dilution was less than 1.5%. The binding spectrum type was determined by the position of wavelengths with the maximum (λmax) and minimum (λmin) absorbance. The absorbance difference (ΔA) was determined from the maximum and the minimum absorbance. A dissociation constant (Kd) was estimated by fitting the Michaelis-Menten equation (eq. 4) to the concentration-dependent absorbance difference data.

Data Analysis. Kinetic data were analyzed using WinNonLin (version 3.2; Pharsight, Mountain View, CA), with proper weighting schemes. Goodness of fit was evaluated by the F-test (for nested models), distribution of residuals, coefficient of variation (CV), and visual inspection of the Eadie-Hofstee plots.

Statistical analysis was performed by NCSS 2001 (NCSS, Kaysville, UT). The Wilcoxon signed-rank test was used for the comparison of metabolite formation and substrate disappearance clearance of paired liver microsomes and kidney microsomes with CYP3A5*1/*3 groups, and the combined loss of both groups (m/z 521.2) displayed similar mass spectral fragmentation patterns (Table 1; the fragmentation pattern for the second peak in Fig. 2E is not shown). Fragments corresponding to the loss of the cyclohexane ring (m/z 826.3), its associated fragments) revealed a mass spectrum with published spectra for tacrolimus metabolites, it was tentatively identified as 13-demethyltacrolimus.

Two additional minor mono-demethylation metabolites were isolated from human liver microsomes (Fig. 2, C and D). The peak shown in Fig. 2C exhibited a parent ion and fragmentation pattern similar to those of 13-DMT (Table 1), but lacked prominent fragments derived from puran to furan rearrangement, suggesting no metabolism on the puran ring. Moreover, the most prominent fragment, corresponding to loss of the cyclohexene ring (m/z 808.3), suggested no change in that part of the molecule. Based on this information and a comparison of its mass spectrum with published spectra for tacrolimus metabolites, it was tentatively identified as 15-demethyl tacrolimus.

The desmethyl metabolite shown in Fig. 2D exhibited ion fragments corresponding to the loss of the cyclohexene (m/z 616.2) and combined loss of the cyclohexene and pepercolate (m/z 475.1) functional groups (and their associated secondary fragments), that retained the same mass as those of the parent compound (Table 1). In addition, inspection of fragments associated with the loss of the pepercolate functional group (m/z 701.4 and its associated fragments) revealed a loss of 14 (CH4) compared with corresponding fragments of the parent compound, as seen for the other demethylated metabolites. Together, these data strongly suggested demethylation on the cyclohexene ring. Based on this information and a comparison of its mass spectrum with published spectra for tacrolimus metabolites, it was identified as 31-desmethyl tacrolimus.

Two hydroxyl metabolites were isolated. Both exhibited an m/z of 842.6 under MS-SIR conditions, corresponding to sodium adducts of the parent ion. Similar to the isomers of 13-DMT, both metabolites gave rise to the same MS-SIR spectra (Fig. 2E) and spontaneous interconversion between them. However, the interconversion rate was much slower compared with that of the two 13-DMT isomers. Both gave an identical fragmentation pattern with MS-multiple reaction monitoring detection (Table 1; the fragmentation pattern for the second peak in Fig. 2E is not shown). Fragments corresponding to the loss of the pepercolate (m/z 730.9) and cyclohexene (m/z 632.3) groups, and the combined loss of both groups (m/z 521.2), displayed a gain of 16 (O) compared with the corresponding fragments of tacrolimus (Table 1), indicating hydroxylation at a different part of the.
molecule. Based on this evidence and a comparison of its mass spectrum with published spectra for tacrolimus metabolites, the two peaks were identified as isomers of 12-hydroxytacrolimus.

**Microsomal Tacrolimus Metabolite Profiles.** Incubation of human liver microsomes with 0.2 μM tacrolimus yielded the expected four primary metabolites, 13-DMT, 15-DMT, 31-DMT, and 12-HT, with 13-DMT generated in the greatest abundance (Fig. 4A). Secondary metabolites were not detected under the relatively short incubation times used. The formation of 13-DMT, 31-DMT, and 12-HT was 1.7-, 4.2-, and 1.8-fold higher for *CYP3A5*/*3 microsomes compared with *3/*3 microsomes (p = 0.007, 0.0002, and 0.002, respectively). In contrast, the formation of 15-DMT was similar between the two genotype groups. Total formation of the four primary tacrolimus metabolites was 1.8-fold higher for *CYP3A5*/*3 than for *3/*3 microsomes (p = 0.006). CL\text{u,HLM} was 1.4 ± 0.3 and 0.9 ± 0.5 ml/min/mg protein for CYP3A5/*1/*3 and *3/*3 microsomes, respectively.

Incubation of tacrolimus (0.2 μM) with human kidney microsomes from a *CYP3A5*/*3 donor yielded predominantly 13-DMT; however, 31-DMT and 12-HT were also detected. The formation of 15-DMT was negligible. In contrast, only 13-DMT was detected after incubation with microsomes from a *CYP3A5*/*1/*3 donor. As shown in Fig. 3B, the formation of 13-DMT was 13.5-fold higher for CYP3A5/*1/*3 microsomes, compared with *3/*3 microsomes (p = 0.03).

**Spectrophotometric Analysis of Tacrolimus Binding to CYP3A4 and CYP3A5.** Tacrolimus exhibited a concentration-dependent type I binding spectrum (λ\text{max} = 390 nm and λ\text{min} = 420 nm) for both rCYP3A4 and rCYP3A5. Both enzymes showed similar affinity to tacrolimus. The apparent K\text{m} was 0.72 ± 0.18 μM for CYP3A4 and 0.90 ± 0.06 μM for CYP3A5 (Fig. 5, A and B). These values were uncorrected for nonspecific protein binding, which could not easily be determined.

**Tacrolimus Metabolite Formation Kinetics.** The same four primary tacrolimus metabolites produced by liver microsomes were also detected in incubations with heterologously expressed CYP3A enzymes. Again, 13-DMT was the dominant metabolite at all substrate concentrations, and it was evaluated further to determine kinetic constants for the reaction. A simple hyperbolic equation provided the best fit to 13-DMT formation from tacrolimus incubations with CYP3A enzymes that were coexpressed with cytochrome b_5 (Fig. 6, A and B). The estimated V\text{max} values were 8.0 and 17.0 nmol/min/nmol P450, and K\text{m,u} values were 0.21 and 0.21 μM for rCYP3A4+b_5 and rCYP3A5+b_5, respectively. Therefore, CL\text{u,HLM} for rCYP3A5+b_5 was more than twice that of rCYP3A4+b_5 (Table 2).

CYP3A enzymes supplemented with cytochrome b_5 displayed complex kinetic behavior for the formation of 13-DMT (Fig. 6, C and D). A two-substrate model yielded the best fit. For rCYP3A4, V\text{max} and K\text{m,1} were 0.1 and 7.8 nmol/min/nmol P450, and K\text{m,2} and K\text{m,2} were 0.2 and
fraction in the incubations was about 20% (see Materials and Methods). The average CL_intrinsic of microsomes with a CYP3A5*1/*3 genotype was 15.9 ± 9.8 ml/min/mg protein, compared with 6.1 ± 3.6 ml/min/mg protein for CYP3A5*3/*3 microsomes; this difference was significant (p = 0.001).

**In Vitro to in Vivo Scaling of Tacrolimus Metabolic Clearance.**

Hepatic plasma clearances calculated from unbound liver microsomal intrinsic clearances, using both substrate disappearance and metabolite formation kinetic approaches, are presented in Table 3. All estimates were very close to the plasma clearance of tacrolimus (1.69 l/h/kg) reported in the literature (Jusko et al., 1995a). Moreover, livers with a CYP3A5*1/*3 genotype, on average, had a higher predicted Cl_H plasma compared with those with a CYP3A5*3/*3 genotype.

Unbound intrinsic formation clearances calculated from tacrolimus incubations with each recombinant enzyme were also used to predict the hepatic clearance, taking into consideration the specific CYP3A4 and CYP3A5 contents of the selected sets of genotyped livers described above. The predicted hepatic clearance values using heterologously expressed CYP3A4 and CYP3A5 prepared with cytochrome b5 and based on the formation of 13-DMT were 0.65 ± 0.11 (well stirred) and 0.83 ± 0.15 (parallel-tube) l/h/kg for CYP3A5*3/*3 liver microsomes, and 0.99 ± 0.10 (well stirred) and 1.20 ± 0.06 (parallel-tube) l/h/kg for CYP3A5*1/*3 liver microsomes. Similarly, the predicted hepatic clearance values calculated from heterologously expressed enzymes supplemented with cytochrome b5 were 0.33 ± 0.09 (well stirred) and 0.38 ± 0.12 (parallel-tube) l/h/kg for CYP3A5*3/*3 liver microsomes, and 0.68 ± 0.15 (well stirred) and 0.86 ± 0.20 (parallel-tube) l/h/kg for CYP3A5*1/*3 liver microsomes. Again, livers with a CYP3A5*1/*3 genotype showed a higher predicted Cl_H plasma compared with those with a CYP3A5*3/*3 genotype.

**Discussion**

The elimination of tacrolimus from the human body is mediated almost exclusively by P450-dependent metabolism, with less than 3% of a dose excreted unchanged in urine (Moller et al., 1999).
Previous investigators have shown that the primary routes of tacrolimus metabolism (Fig. 1) are catalyzed by CYP3A4 and CYP3A5 (Sattler et al., 1992; Shiraga et al., 1994; Lampen et al., 1995; Bader et al., 2000). In this study, we showed that CYP3A5 is a more efficient catalyst of tacrolimus than is CYP3A4. In addition, based on the observed difference in the mean unbound tacrolimus intrinsic disappearance clearance (CLDisapp,uHLM) for microsomes from CYP3A5*3/*3 and CYP3A5*1/*3 donors with matched CYP3A4 contents, CYP3A5 will contribute, on average, approximately 60% of the total tacrolimus metabolism by the liver of a CYP3A5*1 carrier.

We did not evaluate the extent of tacrolimus metabolism by all of the livers in our tissue bank. However, previous analysis of those same livers indicated that CYP3A5 accounted for 46 to 85% of the total CYP3A4 and CYP3A5 content for subjects carrying at least one CYP3A5*1 allele (Lin et al., 2002). This observation is consistent with the respective intrinsic tacrolimus clearance for CYP3A4 and CYP3A5 (data from this study) and interpatient differences in the tacrolimus trough concentration/dose ratios seen in vivo for CYP3A5*1-carrying genotype groups (Hesselink et al., 2003; Thervet...
et al., 2003; Zheng et al., 2003; Haufrlo et al., 2004). Although in the study of Lin et al. (2002), we observed a close concordance between CYP3A4 and CYP3A5 content in CYP3A5*1 carriers, it is possible that some individuals in the transplant population carrying a CYP3A5*1 allele will nonetheless exhibit limited contribution from CYP3A5 to the total tacrolimus metabolic clearance because of a much higher amount of CYP3A4 expression.

As shown in Table 3, the predicted hepatic plasma clearances of tacrolimus (CLₚ⁰Blo×), calculated using liver microsomes and the substrate disappearance approach, were close to the literature-reported value of 1.69 l/h/kg (Jusko et al., 1995a). However, it is important to note that although tacrolimus exhibits a high plasma clearance, reflecting the high efficiency of CYP3A catalysis, the whole blood clearance is low because of the extensive binding of tacrolimus to the blood and other tissue (Jusko et al., 1995a). The CLₚBlo× predicted using a substrate disappearance approach yielded a higher CLₚBlo× compared with that using metabolite formation. Lower predicted values were obtained from the metabolite formation approach. However, this could be due to inaccuracy in determining the concentration of tacrolimus metabolite standards (see Materials and Methods) as well as an incomplete accounting of all tacrolimus metabolites produced by liver microsomes. Nonetheless, the dominant contributions from CYP3A4 and CYP3A5 to the hepatic clearance of tacrolimus were evident from a comparison of CLₚBlo× predicted using kinetic data from heterologously expressed enzymes and liver microsomes, where formation of 13-DMT and 12-HT could explain more than half of the total hepatic tacrolimus clearance and accurately predicted the difference between livers with different CYP3A5 genotypes.

Our experimental findings are fully consistent with differences in the tacrolimus trough concentration/dose ratio reported for kidney, lung, and heart transplant patients with different CYP3A5 genotypes. Hesselink et al. (2003; Thervet et al., 2003; Zheng et al., 2003; Haufrlo et al., 2004). Patients carrying the CYP3A5*1 allele exhibit a higher apparent oral tacrolimus clearance (lower dose-adjusted trough concentration) than those with a CYP3A5*3/*3 genotype. The relative importance of CYP3A5 to the metabolic clearance of tacrolimus has been reported recently. Kamdem et al. (2005) showed that CYP3A5 exhibited a higher Vₘₘₚ and a similar Kₘ for 13-DMT formation compared with CYP3A4. In addition, using liver microsomes, CYP3A5 high expressers exhibited a higher predicted 13-DMT clearance compared with CYP3A5 low expressers. However, their tacrolimus intrinsic clearance estimated using heterologously expressed CYP3A enzymes was much lower (50–70 times) than the unbound intrinsic clearance reported in our study. Although unclear, this discrepancy may be explained by the extensive nonspecific binding of tacrolimus to incubation vessels and incubation protein that we observed and that can greatly complicate kinetic measurements if uncontrolled.

Quantitation of the unbound concentration of tacrolimus ([S]ₜ) in the various incubation systems was critical for accurate prediction of the intrinsic tacrolimus clearance. However, it is also worth noting the relative contribution of nonspecific binding to the experimental apparatus (1 – fₐ) and to the microsomal protein (1 – fₚ). In this case, fₐ varied 5-fold and fₚ was relatively constant over the tacrolimus concentration range. Accordingly, the major factor contributing to the nonlinearity of tacrolimus unbound drug concentration was nonspecific binding of tacrolimus to the experimental apparatus rather than binding to the microsomal protein. The concentration of the microsomal protein used in our experiments was relatively low and the extent of nonspecific binding to the apparatus was very high. With higher microsomal protein, the extent of nonspecific binding to the apparatus could be lower, but this presumably would be offset by greater microsomal binding.

Our experiments with heterologously expressed CYP3A enzymes and liver microsomes showed that 13-DMT was the dominant metabolite produced by CYP3A4 and CYP3A5, consistent with previous observations (Iwasaki et al., 1993). However, the two isoforms exhibited some regioselective differences. Both enzymes generated 13-DMT and 12-HT, although their formations were higher for CYP3A5 than CYP3A4. However, with respect to the other two primary metabolites, CYP3A5 formed predominantly 31-DMT, whereas CYP3A4 formed predominantly 15-DMT. Also, compared with CYP3A5*3/*3 liver microsomes, CYP3A5*1/*3 liver microsomes exhibited much higher 31-DMT formation, moderately higher 13-DMT and 12-HT formation, but similar 15-DMT formation. For all of these comparisons, we acknowledge that uncertainties about the absolute concentrations of our metabolite standards could change the rank order of metabolite production by any given enzyme preparation. However, relative comparison of rates of metabolite formation by different enzyme preparations (e.g., CYP3A4 versus CYP3A5) should be unaffected.

The detection of 12-HT after incubations with CYP3A4 and CYP3A5 differs from previously published results. Using heterologously expressed enzyme, Bader et al. (2000) found that desmethyl tacrolimus could be formed by both enzymes. In contrast, 12-HT was formed only by CYP3A5. The reason for this discrepancy is unknown. However, given the low rate of formation of 12-HT, it is not expected to contribute significantly to the systemic clearance of tacrolimus. Indeed, Mancinelli et al. (2001) found that in a study of healthy subjects, 13-DMT was the major metabolite after oral administration, followed by 15-DMT and 31-DMT. No 12-HT was detected in these subjects.

The presence of cytochrome b₅ has a profound effect on the kinetics of tacrolimus metabolism, as has been observed previously for many CYP3A substrates. Interestingly, the nature of the effect depended on the method of its incorporation into the reaction mixture; i.e., de novo synthesis of b₅ with CYP3A in the transfected insect cell or supplementation to the CYP3A preparation. Presumably, a difference in the way de novo and supplemented cytochrome b₅ interacted with P450 enzymes and reductase contributed to the differences in tacrolimus metabolism kinetics. One obvious and commonly reported effect of cytochrome b₅ was a difference in the concentration-dependent rate of metabolite formation. Rates of 13-DMT formation were higher with rCYP3A4/5 + b₅ compared with rCYP3A4/5 at all substrate concentrations tested. Surprisingly, there was a difference in the kinetic behavior of metabolite formation for the two systems containing cytochrome b₅. Complex kinetics was clearly discernable from the Eadie-Hofstee plots when cytochrome b₅ supplementation was used, but much less so (and not significant) when cytochrome b₅ was coexpressed with CYP3A enzymes. It remains to be determined which expression system is more relevant for in vitro to in vivo predictions. However, regardless of the enzyme system used, CYP3A5 was a superior catalyst of tacrolimus metabolism compared with CYP3A4.

There appears to be a marked difference in penetrance of the CYP3A5 polymorphism with respect to the metabolic clearance of tacrolimus and cyclosporine. A strong dependence of oral tacrolimus clearance on the CYP3A5 genotype is accurately predicted by a 2-fold higher intrinsic clearance of CYP3A5, compared with CYP3A4. In contrast, compared with CYP3A4, the metabolism of cyclosporine by CYP3A5 is more restricted (only one of three primary metabolites observed) and less efficient (intrinsic clearance ~43% of CYP3A4).
The CYP3A5 genotype was also a strong predictor of the metabolism of tacrolimus to 13-DMT in the human kidney, as expected from previous work in our laboratory showing much higher levels of CYP3A5 proteins in kidneys from donors carrying a CYP3A5*1 allele than in CYP3A5*3 homozygotes (Givens et al., 2003). Other P450 isozymes might contribute to the renal elimination of tacrolimus. However, those that have been screened (e.g., CYP1A2, CYP2A6, CYP2B6, CYP2C8, and CYP2C9) had either no detectable or extremely low activity for 13-DMT formation (Kamdem et al., 2005).

In summary, CYP3A5 was a more efficient enzyme for tacrolimus biotransformation than CYP3A4. The two enzymes exhibited similar binding affinities for tacrolimus, and a similar unbound Ka in vivo, but higher Vmax and unbound intrinsic clearance compared with CYP3A4. Liver and kidney microsomes from donors with a CYP3A5*1/*3 genotype had a higher tacrolimus clearance compared with CYP3A5*3/*3 microsomes. Moreover, the predicted plasma clearance of tacrolimus was higher in individuals with a CYP3A5*1/*3 genotype than those with a CYP3A5*3/*3 genotype. These data explain the observed effect of CYP3A4 genotype on systemic tacrolimus disposition and support the contention that prospective genetic testing of a graft recipient might guide the initial tacrolimus dose selection in non-liver transplant patients. In addition, CYP3A5 genotyping might also be of some clinical value in understanding and predicting intrarenal accumulation of tacrolimus and the risk of drug-induced nephrotoxicity.

Acknowledgments. We thank Dr. James D. Perkins from the Department of Surgery, University of Washington for help with acquiring human liver samples.

References


Address correspondence to: Dr. Kenneth E. Thummel, Department of Pharmaceutics, Box 357610, University of Washington, Seattle, WA 98195-7610. Email: thummel@u.washington.edu