Metabolism of Sirolimus in the Presence or Absence of Cyclosporine by Genotyped Human Liver Microsomes and Recombinant Cytochromes P450 3A4 and 3A5

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ABSTRACT:

Sirolimus is an immunosuppressive drug currently used alone or in combination with cyclosporine. Both drugs undergo extensive metabolism by the CYP 3A enzymes. This study aimed at comparing the activity of recombinant CYP (rCYP) 3A4 and 3A5 toward sirolimus, investigating the effect of cytochrome on the metabolic rate of these two cytochromes P450 (P450s), as well as the impact of the CYP 3A5*3 polymorphism on that of human liver microsomes (HLMs). Two distinct approaches were used; i.e., the measurement of (1) hydroxy-sirolimus and desmethyl-sirolimus production, and (2) sirolimus depletion by the in vitro half-life method. rCYP 3A5 exhibited a lower intrinsic clearance (CLint) for both hydroxylation (0.11 versus 0.24 μmol P450/min) and depletion of sirolimus (0.64 versus 2.36 μmol P450/min) than rCYP 3A4. Similar CLint values for hydroxylation, demethylation, and depletion were found when comparing a pool of HLMs carrying at least one CYP 3A5*1 (active) allele with a pool of HLMs not expressing CYP 3A5. This was further confirmed for sirolimus depletion using individual microsome preparations (p = 0.42). A deeper inhibitory effect of cyclosporine on the CLint of sirolimus depletion was found for rCYP 3A4 than for rCYP 3A5 (i.e., −44% versus −8% at 0.62 μM, 750 μg/l cyclosporine), and sirolimus metabolism was slightly less inhibited for HLMs expressing CYP 3A5 than not (−38% versus −56%). In the absence of cyclosporine, the CYP 3A5*3 polymorphism may not influence significantly sirolimus metabolism at the hepatic level. However, strong CYP 3A4 inhibition by cyclosporine could unveil the influence of this polymorphism.

Sirolimus (previously known as rapamycin) is a potent immunosuppressive drug used after renal transplantation. It displays a synergistic action with calcineurin inhibitors but a distinctive mechanism of action and is therefore often used in combination with cyclosporine. Sirolimus is characterized by a highly variable absorption and low bioavailability. Indeed, it is subject to extensive presystemic metabolism by the intestinal (Lampen et al., 1998) and hepatic (Sattler et al., 1992) CYP 3A enzymes. At least 12 first, second, and third generation metabolites have been isolated from various biologic sources or in vitro experiments (Gallant-Haidner et al., 2000). None contributes significantly to the pharmacological activity of sirolimus.

CYP 3A4 hepatic or intestinal content is highly variable between individuals, and inhibition or induction of this isofrom by xenobiotics is common. CYP 3A5 is structurally similar to CYP 3A4 but is polymorphically expressed because of mutations in the CYP 3A5 gene. Its expression is almost fully abolished in homozygous carriers of the mutated CYP 3A5*3 allele (approximately 70% of Caucasian and 50% of Afro-Amerian populations) (Hustert et al., 2001; Kuehl et al., 2001). It displays overlapping substrate specificity with CYP 3A4 but different catalytic activity, higher in some cases (Patki et al., 2003) and lower in others (Kamdem et al., 2005). In addition, there is increasing evidence that CYP 3A4 and CYP 3A5 have a differential susceptibility to enzyme inhibitors (Granfors et al., 2006), so that the CYP 3A5 genotype might be a determinant not only of interindividual variability in the metabolism of CYP 3A substrates, but also of individual susceptibility to drug-drug interactions involving CYP 3A inhibition. As far as sirolimus is concerned, significant associations were found between CYP 3A5 genotypes and dose requirement in patients without calcineurin inhibitor (Anglicheau et al., 2005; Le Meur et al., 2006), but not in patients coadministered either cyclosporine or tacrolimus (Anglicheau et al., 2005). Sirolimus, tacrolimus, and cyclosporine are all substrates of the CYP 3A enzymes and P-glycoprotein drug transporter. This can lead to pharmacokinetic interactions, as initially described in rats (Stepkowski et al., 1996). In renal transplant patients, coadministration of the two drugs resulted in increased sirolimus area under the concentration curve (1.45-fold), through concentration (1.49-fold) and maximal concentration (1.72-fold), compared with staggered administration, with no significant consequence on cyclosporine exposure (Kaplan et al., 1998). This interaction mainly involves CYP 3A inhibition as demonstrated in rats (Wacher et al., 2002) as well as in vitro using CYP 3A4-transfected Caco-2 cells (Cummins et al., 2004). However, there has been no study of the particular involvement of CYP 3A5 in, and none investigating the consequence of its polymorphism on, the cyclosporine/sirolimus interaction.

ABBREVIATIONS: HLM, human liver microsome; P450, cytochrome P450; rP450 recombinant cytochrome P450; TFC-MS/MS: turbulent flow chromatography-tandem mass spectrometry; CLint, intrinsic clearance; MRM, multireaction monitoring; SNP, single nucleotide polymorphism.
The aim of the present study was therefore to compare the metabolic rates of sirolimus by CYP 3A4 and CYP 3A5 using recombinant enzymes as well as human liver microsomes genotyped for CYP 3A5*,3, and investigate the differential inhibitory effect of cyclosporine on these two metabolic pathways.

Materials and Methods

Chemicals. NADPH, troleandomycin (TAO), proteinase K, and SDS were purchased from Sigma-Alrich (St. Louis, MO). Sirolimus was obtained from Wyeth Ayerst (Pearl River, NY) and cyclosporine A from Novartis Pharma AG (Basel, Switzerland). Taqman PCR Universal Master Mix was purchased from Applied Biosystems (Courtaboeuf, France). All chemicals and reagents used for DNA extraction and genotyping were of biological molecular grade; others were of analytical grade.

Pooled Human Liver Microsomes and Recombinant P450. Pooled human liver microsomes (HLMs), microsomes prepared from baculovirus-infected insect cells (Supersomes) expressing human CYP 3A4 or 3A5 (recombinant P450) and controls were purchased from BD Gentest (Woburn, MA). Neither CYP 3A4 nor CYP 3A5 was coexpressed with cytochrome b5, and controls were purchased from Applied Biosystems (Courtaboeuf, France). All chemicals and reagents were of biological molecular grade; others were of analytical grade.

Human Liver Microsomes Genotyped for CYP 3A5*. Thirty-two human liver samples derived from surgical specimens were obtained from Biopredic International (Rennes, France). All samples were collected after donors had given their informed consent, in accordance with the French bioethics laws.

Genomic DNA extraction. Genomic DNA was extracted from human liver samples as follows: approximately 10 mg of liver tissue was ground briefly in a 1.5-ml tube containing 200 μl of extraction buffer (7.5 mM NaCl, 2.4 mM EDTA, 37.3 mM SDS, and 0.81 mg/ml Proteinase K) using a motor-driven pestle (Kontes Glass Company, Vineland, NJ). A further aliquot (910 μl of extraction buffer) was added to the tube and the mixture incubated for 90 min at 55°C. After addition of 300 μl of 6 M NaCl and centrifugation, the supernatant was collected and DNA precipitated with 99% ethanol. After centrifugation, the pellet was washed with 1 ml of 70% ethanol and finally resuspended in 200 μl of a buffer containing 1 mM Na3EDTA and 1 M phosphate buffer, pH 7.5 (Tris-HCl (5:95)).

Identification of genotypes. Liver samples were genotyped for the CYP 3A5*3 single nucleotide polymorphism (SNP) 6986A>G (reference SNP 776746), using real-time quantitative polymerase chain reaction based on the 5’ nucleic acid allelic discrimination assay (ABI PRISM 7800 Sequence Detection System; Applied-Biosystems, Courtaboeuf, France). Primers and probes were as follows: forward primer, CGAAAGTCTCCTAGCTTCTTCTAACA; reverse primer, TGAAGGGAAGTCTGTCCTCAAACAG; wild-type probe, TTTTGCTTCATATCTC; mutated probe, TTTTGCTTCATAGTCTC. They were designed on the basis of the sequence of the CYP 3A5*3 gene using Primer Express 2.0 software (Applied Biosystems) and purchased from Applied Biosystems. In brief, 1 to 20 ng of genomic DNA were mixed with the “assay-by-design” (Applied Biosystems) and PCR Master Mix in a total volume of 14 μl and submitted to PCR with standard thermal cycler parameters. The distribution of the CYP 3A5*3 allele in the 32 liver specimens was compared with the theoretical distribution of Hardy-Weinberg using the Fisher’s exact test.

Preparation of microsomes. Microsomes were prepared individually from the 32 liver samples by differential centrifugation as described previously (Picard et al., 2004). Protein concentration of the microsomal suspensions were measured according to the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Enzyme Incubation Conditions. Standard incubation mixture consisted of 0.1 M Tris buffer pH 7.4, 10 mM MgCl2, 2 mM NADPH, HLMs (0.1 mg protein/ml) or rP450 (10–50 pmol/ml), and substrate (sirolimus) with or without inhibitor (cyclosporine) prepared in acetonitrile/water (50:50 v/v) (1.25% acetonitrile in final incubation medium). Incubations were carried out at 37°C. Sirolimus and microsomes were preincubated for 5 min before the reaction was initiated by adding NADPH. Incubations were stopped after 10 min with ice-cold acetonitrile (v/v). In pilot experiments, control incubations of sirolimus were conducted without microsomes, NADPH, or both; sirolimus disappearance was NADPH- and microsome-dependent. Unless otherwise mentioned, incubation experiments were performed in triplicate.

Chemical Inhibition Experiments. To evaluate the effect of enzyme inactivation by the CYP 3A mechanism-based inhibitor TAO, pooled HLMs were preincubated in the presence of 1 mM TAO at 37°C for 15 min. The reaction was then initiated by adding 0.11 μM (100 μg/ml) sirolimus and a further aliquot of NADPH, and stopped after 10 min by 200 μl of ice-cold acetonitrile. Incubations were performed in triplicate, in parallel with control incubation without TAO. The concentrations of sirolimus and metabolites after incubation with TAO (n = 3) were compared with those of the control experiment and percentage inhibition was calculated.

Enzyme Kinetic Experiments. In preliminary experiments, linearity of metabolite formation with increasing microsomal protein concentration (0.1–1 mg/ml), rP450 concentration (10–100 pmol/ml), and incubation time (10–60 min) was checked. Kinetic experiments were then performed by incubation of increasing sirolimus concentrations (0.11–16.41 μM) using 0.1 mg/ml microsomal protein, 10 pmol/ml rCYP 3A4, or 20 pmol/ml rCYP 3A5, 10 min incubation time, and a total volume of 200 μl.

Determination of Sirolimus Depletion Using HLMs, rCYP 3A4, and rCYP 3A5. In substrate depletion experiments, the disappearance of sirolimus (initial concentration 0.11 μM) with incubation time was monitored. Incubations were performed as described above in a total volume of 600 μl. Aliquots (50 μl) were removed at 0, 2, 5, 10, 15, 20, and 30 min and added to 50 μl of ice-cold acetonitrile to stop the reaction. To allow accurate estimation of sirolimus disappearance even after a short incubation time, rP450 concentration was set at 20 pmol/ml (rCYP 3A4) and 50 pmol/ml (rCYP 3A5).

Interaction Experiments. The effect of increasing concentration of cyclosporine (0.42–4.16 μM, 500–5000 μg/ml) on sirolimus metabolism by pooled HLMs was evaluated after the general incubation procedure described above in triplicate experiments. The concentration of cyclosporine inducing 50% inhibition of sirolimus depletion with respect to a cyclosporine-free control (IC50) was estimated by nonlinear regression of the log-percentage of cyclosporine concentration versus percentage inhibition (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Then, the effect of a single cyclosporine concentration (0.62 μM, 750 μg/l), approximately half the IC50 on sirolimus depletion by genotyped HLMs, on rCYP 3A4 and rCYP 3A5 was evaluated following the procedure described above.

Analytical Methods. Sirolimus determination. Sirolimus determination was performed using a turbulent flow chromatography-tandem mass spectrometry system. The system consisted of a Cohesive 2300 system (Cohesive Technologies, Milton Keynes, UK) equipped with a CTC HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) kept at 6°C, two binary high-pressure Agilent 1100 pumps (Agilent Technologies, Palo Alto, CA), and three six-port switching valves controlled by the Aria OS software package (Cohesive Technologies, Franklin, MA). System configuration, parameters, and analytical procedures were previously described in details (Sauvage et al., 2006). In brief, online extraction was performed at a high flow rate (1.25 ml/min) on a Cyclone, 50-μm particle size (50 × 0.5 mm i.d.) column (Cohesive Technologies, under alkaline conditions [phase A1, 20 mM ammonium acetate in water, 35% acetonitrile (v/v) and 1% ammonium; phase B1, mixture of acetone/acetonitrile/propylene glycol-2 (50:30:20; v/v/v)]. Chromatographic separation was performed under acidic conditions (phase A2, 0.1% formic acid in water and phase B2, 0.1% formic acid in acetonitrile) using an Xterra MS C18, 5-μm (20 × 2.1 mm i.d.) column (Waters, Milford, MA) kept at 65°C, with a constant flow rate of 200 μl/min.

Detection was performed with a TQ Quantum Discovery tandem mass spectrometry system (Thermo Electron, Les Ulis, France) equipped with an orthogonal electrospray ionization source and controlled by the Xcalibur computer program. Sirolimus exhibited an m/z of 931.3 corresponding to ammonium adduct of the parent ion. Tandem mass spectrometry detection was performed in the positive ion, multiple reaction monitoring (MRM) mode using two transitions for sirolimus (m/z 931.3→882.1; m/z 931.3→864.1) and for the internal standard desmethoxysirolimus (m/z 901.3→816.4; m/z 901.3→834.2).

The method was fully validated for sirolimus determination in blood samples and adapted to in vitro incubation supernatant. In brief, 100 μl of incubation supernatant was added to 150 μl of a mixture of methanol/aqueous zinc sulfate (70:30 v/v) and 50 μl of internal standard (100 μg/l in acetonitrile). The mixture was vortex-mixed for 15 s and introduced in a 200-μl vial for injection. Calibration standards at 0, 1, 2.5, 5, 10, 25, 50, and 100 μg/l and internal quality controls at 1, 10, and 100 μg/l were prepared by spiking blank
Sirolimus depletion of 898.3 /H11006 capacity for hydroxy-sirolimus formation, resulting in $CL_{\text{int}}$ (parameters are shown in Table 1. HLMs showed a higher affinity and correctly fitted by the Michaelis-Menten equation (Fig. 2). Kinetic experiments).

### Data Analysis

Microsomal kinetic data were model-fitted and apparent $K_m$ and $V_{\text{max}}$ calculated according to the Michaelis-Menten model using Winreg 3.1 software (Dr J. Debord, Limoges University Hospital, Limoges, France).

The in vitro half-life of total sirolimus depletion ($t_{1/2}$) in HLMs, rCYP 3A4, and rCYP 3A5 was calculated using the formula $t_{1/2} = 0.693/k$, where $k$ represents the slope of the linear regression line between the log-percentage of substrate remaining versus the incubation time. The total in vitro intrinsic clearance ($CL_{\text{int}}$) was then estimated using the following equation (Obach, 1999): $CL_{\text{int}} = k l/[\text{microsomal protein} \times rP450]$.

### Results

**Sirolimus Metabolism by Pooled HLMs.** Several compounds with ion transitions compatible with those expected for hydroxy, dihydroxy, trihydroxy, desmethyl, di-desmethyl, and hydroxy-desmethyl metabolites of sirolimus were found (data not shown). None was detected in control incubations. The identity of the two main metabolites detected was confirmed by their product ion spectra, which showed $m/z$ 947.1→898.1 and 947.1→880.1 for hydroxy-sirolimus, and $m/z$ 917.1→868.1 and 917.1→950.1 for desmethyl-sirolimus. Due to the lack of pure compound, metabolite concentrations were estimated with respect to the sirolimus calibration curve and expressed in molar equivalents of sirolimus.

**Identification and determination of sirolimus metabolites.** Sirolimus metabolites were investigated using the turbulent flow chromatography-tandem mass spectrometry method described above except that mass spectrometric detection was performed in the Q3-only full-scan mode between $m/z$ 800 and $m/z$ 1000. Metabolite structures were confirmed in the product ion scan mode by comparing their fragmentation pattern with that of sirolimus. Two MRM transitions were selected for quantification of each of the two main metabolites identified, $m/z$ 947.1→898.1 and $m/z$ 947.1→880.1 for hydroxy-sirolimus, and $m/z$ 917.1→868.1 and $m/z$ 917.1→950.1 for desmethyl-sirolimus. Due to the lack of pure compound, metabolite concentrations were estimated with respect to the sirolimus calibration curve and expressed in molar equivalents of sirolimus.

Following our incubation conditions, sirolimus metabolism by pooled HLMs was CYP 3A dependent: inactivation of the CYP 3A by TAO resulted in a 99.0% decrease of sirolimus depletion rate. During incubation, sirolimus concentrations followed a monoeponential decay over time (Fig. 2), leading to an estimated $CL_{\text{int}}$ of sirolimus depletion of 898.3 ± 60.9 $\mu$mol/mg protein/min ($n = 3$ experiments).

Hydroxy-sirolimus and desmethyl-sirolimus formation rates were correctly fitted by the Michaelis-Menten equation (Fig. 2). Kinetic parameters are shown in Table 1. HLMs showed a higher affinity and capacity for hydroxy-sirolimus formation, resulting in $CL_{\text{int}}$ ($V_{\text{max}}/K_m$) values approximately twice that of demethylation (Table 1). The sum of the formation $CL_{\text{int}}$ of these two metabolites (96.1 $\mu$mol/mg protein/min) represented approximately 10% of the total $CL_{\text{int}}$ of sirolimus depletion estimated by the in vitro half-life method.

**Metabolism of Sirolimus by rCYP 3A4 and rCYP 3A5.** rCYP 3A4 and rCYP 3A5 showed the same metabolic profile as pooled HLMs, with hydroxy-sirolimus and, to a lesser extent, desmethyl-sirolimus being the main metabolites for both P450s at all substrate concentrations. Hydroxy-sirolimus formation data fitted the Michaelis-Menten model, but desmethyl-sirolimus production was linear up to the highest concentration of substrate used. Thus, the kinetic parameters could only be calculated for hydroxy-sirolimus (Table 1).

As already observed with HLMs, hydroxylation of sirolimus by
The CLint values of sirolimus depletion and kinetic parameters of patient numbers 27, 195, 4, 609, and 0, 195, respectively; these frequencies are in Hardy-Weinberg equilibrium (calculated from heterozygous, and none from homozygous wild-type carriers. Metabolism.

3A5 (Table 2).

sirolimus depletion was 3.5-fold higher for rCYP 3A4 than for rCYP 3A5 (Table 1). Similarly, the estimated CLint of sirolimus hydroxylation (\( V_{\text{max}}/K_{m} \)) was approximately 2-fold higher that of rCYP 3A5 (Table 1). The apparent \( K_{m} \) values of sirolimus depletion by pooled HLMs (Table 1). Although rCYP 3A5 showed a higher affinity for sirolimus than rCYP 3A4, its \( CL_{\text{int}} \) of hydroxylation (\( V_{\text{max}}/K_{m} \)) was approximately 2-fold higher than that of rCYP 3A5 (Table 1). Similarly, the estimated \( CL_{\text{int}} \) of sirolimus depletion was 3.5-fold higher for rCYP 3A4 than for rCYP 3A5 (Table 2).

Effect of the CYP 3A5*3 Polymorphism on Sirolimus Hepatic Metabolism. Among the 32 microsomal preparations used, 27 (84.3%) came from homozygous carriers of CYP 3A5*3^3, five (15.6%) from heterozygous, and none from homozygous wild-type carriers. These frequencies are in Hardy-Weinberg equilibrium (calculated patient numbers 27, 195, 4, 609, and 0, 195, respectively; \( p > 0.099 \). The \( CL_{\text{int}} \) values of sirolimus depletion and kinetic parameters of sirolimus hydroxylation and demethylation were determined using two pools of microsomes, one composed of the microsomes associated with a CYP 3A5*1/*3 genotype and one of those with the CYP 3A5*3/*3 genotype. No difference was found in the \( CL_{\text{int}} \) of sirolimus depletion, hydroxylation, or demethylation between the two microsome pools (Tables 1 and 2). This was statistically confirmed by incubation of sirolimus at 0.11 \( \mu M \) (100 \( \mu g/l \)) with the 32 individual microsomal preparations separately: sirolimus mean depletion rates were 84.4 ± 11.6 and 76.3 ± 16.4 pmol/mg/min for microsomes associated with a CYP 3A5*1/*3 or a CYP 3A5*3/*3 genotype, respectively (\( p = 0.42 \)). Metabolite formation rates could not be compared because concentrations were below the limit of detection of the analytical method in most samples.

Effect of Cyclosporine on the Metabolism of Sirolimus by Human Liver Microsomes and rCYP 3A4 and 3A5. Cyclosporine inhibited the metabolism of sirolimus (0.11 \( \mu M \)) in pooled HLMs: inhibition of sirolimus depletion reached 74.5 ± 5.1% (\( n = 3 \) experiments) for 4.16 \( \mu M \) (5000 \( \mu g/l \)) of cyclosporine with an IC\(_{50}\) of 1.08 ± 0.15 \( \mu M \) (Fig. 3). Metabolites could hardly be determined after inhibition by cyclosporine. Thus, inhibition of sirolimus hydroxylation or demethylation could not be evaluated accurately.

The effect of cyclosporine at a concentration approximately half the IC\(_{50}\) (i.e., 0.62 \( \mu M \)) was evaluated on the \( CL_{\text{int}} \) of sirolimus depletion by rCYP 3A4, rCYP 3A5, and individual genotyped microsome preparations. Cyclosporine caused a 44% decrease of the \( CL_{\text{int}} \) of sirolimus depletion by rCYP 3A4, versus only 8% inhibition of rCYP 3A5. Inhibition was 1.5-fold higher in microsomes with the CYP 3A5*3/*3 genotype compared with those with the CYP 3A5*1/*3 genotype (Table 2).

Discussion

After in vitro incubation with recombinant CYP 3A4 or CYP 3A5, as well as human liver microsomes, the major metabolites detected were a hydroxylated and a demethylated metabolite. This is consistent with the finding of Streit et al. (1996), who showed that hydroxy-sirolimus and desmethyl-sirolimus were the main sirolimus metabolites in trough plasma samples from renal transplant patients. We did not perform formal structural identification, but it can be hypothesized that these two metabolites were 12-hydroxy-sirolimus and 39-O-desmethyl-sirolimus, previously reported as the two main metabolites produced by HLMs (Streit et al., 1996). These two metabolites showed low formation rates accounting for approximately 10% of sirolimus depletion by pooled HLMs. Sirolimus is indeed subject to an extensive oxidative metabolism leading to at least 12 identified metabolites (Jacobsen et al., 2001), some of which are further metabolized. Therefore, sirolimus metabolism was further investigated indi-

![FIG. 2. Representative plots showing sirolimus disappearance rate versus time (Top) and metabolite generation rates versus sirolimus concentration (bottom) obtained using pooled human liver microsomes. Each data point represents the average rate from triplicate incubations. Solid lines represent simulated curves generated from the respective model.](Image)

### TABLE 1

<table>
<thead>
<tr>
<th>Hydroxylation of Sirolimus</th>
<th>Demethylation of Sirolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{m} ) (( \mu M ))</td>
<td>( V_{\text{max}} (\mu M) )</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>rCYP 3A4</td>
<td>2.2 (1.8; 2.8)</td>
</tr>
<tr>
<td>CYP 3A5</td>
<td>1.5 (1.3; 1.9)</td>
</tr>
<tr>
<td>Pooled HLMs</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>CYP 3A5*3/*3 HLMs</td>
<td>2.0 (2.2; 1.8)</td>
</tr>
<tr>
<td>CYP 3A5*1/*3 HLMs</td>
<td>1.9 (1.8; 1.9)</td>
</tr>
</tbody>
</table>

N.D., not determined (nonsaturable metabolite formation).

\( ^{a} \) Units are pmol/pmol P450/min for rP450 and pmol/mg protein/min for microsomes.

\( ^{b} \) Units are \( \mu M\) P450/min for rP450 and \( \mu M\) mg protein/min for microsomes.
allele carriers (Hesselink et al., 2003; Thervet et al., 2003). Studies demonstrated that patients with the CYP 3A5*3/*3 polymorphism, and sirolimus is dependent on rCYP 3A5 activity. However, in renal transplant patients, the in vivo clearance of both tacrolimus and sirolimus was independent of rCYP 3A5 activity, since neither metabolite depletion of sirolimus (0.11 ± 0.05) nor tacrolimus (0.80 ± 0.05) was significantly influenced. How-ever, in the present results concerning the hepatic metabolism of sirolimus, it can be hypothesized that the relation between CYP 3A5 polymorphism and sirolimus bioavailability observed in vivo might be due to CYP 3A5 activity in the intestinal wall: saturating concentrations are more likely to be encountered in enterocytes than in hepatocytes or systemic blood. In these circumstances, expression of CYP 3A5, as an isoenzyme, would be of greater importance for sirolimus pre-systemic metabolism. This also suggests that the liver donors’ CYP 3A5*3/*3 genotype would be of minor importance for sirolimus pharmacokinetics compared with that of the recipients.

Cyclosporine was found to inhibit the in vitro metabolism of sirolimus by HLMs. A pharmacokinetic interaction has already been reported between calcineurin inhibitors and sirolimus (Kaplan et al., 1998; Cattaneo et al., 2004). Its mechanism mainly involves inhibition of the CYP 3A isoenzymes and, to a minor extent, inhibition of P-glycoprotein (Wacher et al., 2002; Cummins et al., 2004). Here, the interaction was investigated using the in vitro metabolic half-life method. As stated before, sirolimus follows many different, parallel, and successive metabolic pathways. The major metabolites hydroxy-sirolimus and desmethyl-sirolimus were tentatively used as markers for this interaction study, but both metabolites were produced in such small amounts that they could hardly be determined after CYP 3A inhibition by cyclosporine. Indeed, no inhibition could be detected with a concentration of cyclosporine lower than 0.83 μM (1000 μg/l). Alternatively, the in vitro half-life method indirectly takes into account all metabolic pathways and seems more “physiological” and adequate to predict metabolism modulations in vivo. It allowed us to conduct experiments with low concentration of sirolimus (100 μg/l; i.e., approximately 10 times those observed in patients’ plasma at trough level, but in the range of the maximal concentration achieved after standard dosing and probably lower than pre-systemic plasma and

FIG. 3. Effect of increasing concentration of cyclosporine (0.42–4.16 μM) on the depletion of sirolimus (0.11 μM) in pooled human liver microsomes. Activities are relative to the control rate. Data are given as the mean of three experiments ± S.D.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Mean intrinsic clearance of sirolimus depletion with and without cyclosporine (and inhibition percentage) in rCYP 3A4 and rCYP 3A5 and in two pools of human liver microsomes genotyped for the CYP 3A5*3 single nucleotide polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clint of Sirolimus Depletion</td>
<td>Without Cyclosporine</td>
</tr>
<tr>
<td>rCYP 3A4</td>
<td>2.36 (2.82; 1.90)</td>
</tr>
<tr>
<td>rCYP 3A5</td>
<td>0.64 (0.49; 0.79)</td>
</tr>
<tr>
<td>Microsomes CYP 3A5*3/*3</td>
<td>1277 ± 63</td>
</tr>
<tr>
<td>Microsomes CYP 3A5*1/*3</td>
<td>1265 ± 223</td>
</tr>
</tbody>
</table>

<sup>a</sup> Units are μl/pmol P450/min for rP450 and μl/mg protein/min for microsomes.

<sup>b</sup> Median (minimum; maximum).
hepatic concentrations). To allow unbiased evaluation of the interaction, we used cyclosporine at a concentration lower than the IC_{50} and compatible with the concentrations achieved clinically (i.e., 750 μg/l, which is in the range of cyclosporine blood concentrations 2 h after standard dosing in renal transplant patients). The resulting ratio between the in vitro concentration of sirolimus and cyclosporine (i.e., 1/7.5) was close to or higher than the concentration ratio expected in the blood of patients receiving such a drug combination. Using this method, cyclosporine had a deeper inhibitory effect on sirolimus metabolism by rCYP 3A4 than by rCYP 3A5. Inhibition was also weaker in HLMs derived from expressers of CYP 3A5 compared with nonexpressers. It seems that the influence of the CYP 3A5 polymorphism is dependent on CYP 3A4 inhibition by cyclosporine. Differential inhibition of the CYP 3A4- and 3A5-mediated 6β-hydroxylation of testosterone (a CYP 3A probe substrate) has already been reported for human immunodeficiency virus protease inhibitors: whereas ritonavir, nelfinavir, and saquinavir are nonselective inhibitors of CYP 3A4 and 3A5, indinavir and amprenavir preferentially inhibit CYP 3A4. Gibbs et al. (1999) also showed that CYP 3A5 was less susceptible to inhibition by ketoconazole and fluconazole than CYP 3A4 and suggested that this may contribute to interindividual variability in the magnitude of drug-drug interaction of CYP 3A substrates. Although our results are restricted to the in vitro study of sirolimus metabolism at the hepatic level, it could be hypothesized that the difference in sirolimus exposure between CYP 3A5 expressers and nonexpressers could be deeper in the presence of cyclosporine, since the proportion of CYP 3A5 metabolism may be increased in the liver. To allow unbiased evaluation of the interaction, we used cyclosporine at a concentration lower than the IC_{50} and compatible with the concentrations achieved clinically (i.e., 750 μg/l, which is in the range of cyclosporine blood concentrations 2 h after standard dosing in renal transplant patients). The resulting ratio between the in vitro concentration of sirolimus and cyclosporine (i.e., 1/7.5) was close to or higher than the concentration ratio expected in the blood of patients receiving such a drug combination. Using this method, cyclosporine had a deeper inhibitory effect on sirolimus metabolism by rCYP 3A4 than by rCYP 3A5. Inhibition was also weaker in HLMs derived from expressers of CYP 3A5 compared with nonexpressers. It seems that the influence of the CYP 3A5 polymorphism is dependent on CYP 3A4 inhibition by cyclosporine. Differential inhibition of the CYP 3A4- and 3A5-mediated 6β-hydroxylation of testosterone (a CYP 3A probe substrate) has already been reported for human immunodeficiency virus protease inhibitors: whereas ritonavir, nelfinavir, and saquinavir are nonselective inhibitors of CYP 3A4 and 3A5, indinavir and amprenavir preferentially inhibit CYP 3A4. Gibbs et al. (1999) also showed that CYP 3A5 was less susceptible to inhibition by ketoconazole and fluconazole than CYP 3A4 and suggested that this may contribute to interindividual variability in the magnitude of drug-drug interaction of CYP 3A substrates. Although our results are restricted to the in vitro study of sirolimus metabolism at the hepatic level, it could be hypothesized that the difference in sirolimus exposure between CYP 3A5 expressers and nonexpressers could be deeper in the presence of cyclosporine, since the proportion of CYP 3A5 metabolism may be increased in the liver.

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References


