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 P450 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 and depletion of sirolimus (0.11 versus 0.24 μl/pmol 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 microsone 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 isoform 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-American 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 (Anglicateau 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 (Stepkowsi 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 3AS 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-Aldrich (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 (Courtabeuf, 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 CYP3A4 or 3A5 (recombinant P450) and controls were purchased from BD Gentest (Woburn, MA). Neither CYP 3A4 nor CYP 3A5 was coexpressed with cytochrome P450 (bcl2) and controls were purchased from BD Gentest (Woburn, MA). Human Liver Microsomes Genotyped for CYP 3A5*3. Thirty-two human liver samples derived from surgical specimens were obtained from Biopredic (Saint-Ouen, France). Liver samples were obtained from male and female donors aged 30–80 years old. All donors were in good health and had no history of drug use. Informed consent was obtained from all donors, in accordance with the French bioethics laws. DNA extraction and genotyping were performed using the Aria OS software package (Cohesive Technologies, Franklin, MA). System configuration, parameters, and analytical processes were fully validated for sirolimus determination in blood samples (Cohesive Technologies, Franklin, MA). System configuration, parameters, and analytical processes were fully validated for sirolimus determination in blood samples.
incubation supernatant with sirolimus. The limit of quantitation was 1 μg/l, and calibration curves obtained using quadratic regression from the limit of quantitation up to 100 μg/l yielded $r^2 > 0.998$. The method showed good interassay precision and accuracy with relative standard deviation values ranging from 0.8 to 9.5% and mean relative error from −2.8 to 1.5% over the linearity range. Intra-assay precision and accuracy were assessed by analyzing incubation medium spiked at four concentration levels (1, 5, 25, and 100 μg/l) on the same day and were also satisfactory, with relative standard deviation values ranging from 3.2 to 10.5% and mean relative error always less than 10%.

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.

Data Analysis. Microsomal kinetic data were model-fitted and apparent $K_m$ and $V_{max}$ 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$_{int}$) was then estimated using the following equation (Obach, 1999): CL$_{int}$ = $k$[microsomal protein or rP450].

Results

Sirolimus Metabolism by Pooled HLMs. Several compounds with ion transitions compatible with those expected for hydroxy, dihydroxy, trihydroxy, desmethyl, didesmethyl, and hydroxydesmethyl 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 and 917, consistent with hydroxylation (m/z 931 + 16) and demethylation (m/z 931 - 14) of sirolimus, respectively. Most other fragment ions were also shifted by +16 or −14 U, with respect to those of sirolimus (Fig. 1). The sites of hydroxylation and demethylation were not investigated.

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. As already observed with HLMs, 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, desmethylsirolimus 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. (84.3%) came from homozygous carriers of Metabolism. Among the 32 microsomal preparations used, 27 3A5 (Table 2). sirolimus depletion was 3.5-fold higher for rCYP 3A4 than for rCYP 3A5 (Table 1). Similarly, the estimated CLint of hydroxylation (\(V_{\text{max}}/K_m\)) of rCYP 3A4 and rCYP 3A5 were close to that observed with pooled microsomes (Table 1). Similarly, the estimated CLint 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, 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 CLint 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 CLint 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 \(\mu M\) 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 \(\mu M/l\)) of cyclosporine with an IC50 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 IC50 (i.e., 0.62 \(\mu M\)) was evaluated on the CLint of sirolimus depletion by rCYP 3A4, rCYP 3A5, and individual genotyped microsome preparations. Cyclosporine caused a 44% decrease of the CLint 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-

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Hydroxylation of Sirolimus</th>
<th>Demethylation of Sirolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m)</td>
<td>(V_{\text{max}}) (a)</td>
</tr>
<tr>
<td>rCYP 3A4</td>
<td>2.2 (1.8; 2.8)</td>
<td>0.53 (0.58; 0.48)</td>
</tr>
<tr>
<td>rCYP 3A5</td>
<td>1.5 (1.3; 1.9)</td>
<td>0.16 (0.16; 0.17)</td>
</tr>
<tr>
<td>Pooled HLMs</td>
<td>2.9 ± 0.5</td>
<td>180.0 ± 9.7</td>
</tr>
<tr>
<td>CYP 3A5*2/*3 HLMs</td>
<td>2.0 (2.2; 1.8)</td>
<td>135.2 (134.7; 130.4)</td>
</tr>
<tr>
<td>CYP 3A5*1/*3 HLMs</td>
<td>1.9 (1.8; 1.9)</td>
<td>139.5 (120.0; 158.9)</td>
</tr>
</tbody>
</table>

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

\(b\) Units are \(\mu M\) for rP450 and \(\mu M\) for microsomes.
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

<table>
<thead>
<tr>
<th>CYP 3A</th>
<th>Without Cyclosporine</th>
<th>With 0.62 μM Cyclosporine</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCYP 3A4</td>
<td>2.36 (2.82; 1.90)</td>
<td>1.29 (1.39; 1.19)</td>
<td>44 (51; 37)</td>
</tr>
<tr>
<td>rCYP 3A5</td>
<td>0.64 (0.49; 0.79)</td>
<td>0.62 (0.55; 0.67)</td>
<td>8 (0; 15)</td>
</tr>
<tr>
<td>Microsomes CYP 3A5*1/*3</td>
<td>1277 ± 63</td>
<td>562 ± 35</td>
<td>32 (56; 59)*</td>
</tr>
<tr>
<td>Microsomes CYP 3A5*2/*3</td>
<td>1265 ± 223</td>
<td>784 ± 166</td>
<td>32 (31; 49)*</td>
</tr>
</tbody>
</table>

* Units are μmol/mg protein/min for rP450 and μl/mg protein/min for microsomes.
* Median (minimum; maximum).

**TABLE 2**

### 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.

**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.

**RESULTS**

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 presystemic plasma and
hepatic concentrations). To allow unbiased evaluation of the interaction, we used cyclosporine at a concentration lower than the IC50 for 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-hydroxyla-
tion 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 former. However, Anglicheau et al. (2005) did not find any exposure relationship in patients coadministered sirolimus and cyclosporine, but their group was small (n = 20). In summary, using two different approaches, it was demonstrated that CYP 3A4 was a more efficient catalyst of sirolimus metabolism than CYP 3A5 and that cyclosporine had a deeper inhibitory effect on recombinant CYP 3A4 than on CYP 3A5. Our results also suggest that the CYP 3A5 polymorphism has a limited influence on sirolimus hepatic metabolism, but that it may be enhanced by inhibition of CYP 3A4 by cyclosporine.

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References


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