METABOLISM OF SIROLIMUS IN THE PRESENCE OR ABSENCE OF CYCLOSPORINE BY GENOTYPED HUMAN LIVER MICROSONOMES AND RECOMBINANT CYTOCHROMES P450 3A4 AND 3A5


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Running title:

METABOLISM OF SIROLIMUS BY CYP 3A4 AND CYP 3A5

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Abbreviations: HLM: human liver microsomes; CYP: cytochromes P450; rCYP recombinant cytochromes P450; TFC-MS/MS: turbulent flow chromatography-tandem mass spectrometry; CLint: intrinsic clearance; MRM: Multi-reaction monitoring; SNP: single nucleotid polymorphism
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 cyclosporine on the metabolic rate of these two CYPs, as well as the impact of the CYP 3A5*3 polymorphism on that of human liver microsomes (HLM). Two distinct approaches were used, i.e. the measurement of: (i) hydroxy-sirolimus and desmethyl-sirolimus production; and (ii) sirolimus depletion by the in vitro half-life method. rCYP 3A5 exhibited a lower intrinsic clearance (CL_int) for both hydroxylation (0.11 vs 0.24 µl/pmol CYP/min) and depletion of sirolimus (0.64 vs 2.36 µl/pmol CYP/min) than rCYP 3A4. Similar CL_int of hydroxylation, demethylation and depletion were found when comparing a pool of HLM carrying at least one CYP 3A5*1 (active) allele with a pool of HLM not expressing CYP3A5. This was further confirmed for sirolimus depletion using individual microsome preparations (p=0.42). A deeper inhibitory effect of cyclosporine on the CL_int of sirolimus depletion was found for rCYP 3A4 than for rCYP 3A5 (i.e. -44 % vs -8 % at 0.62 µM, i.e.750 µg/l cyclosporine) and sirolimus metabolism was slightly less inhibited for HLM expressing CYP 3A5 than not (-38 % vs -56%). In the absence of cyclosporine, the CYP 3A5*3 polymorphism may not influence significantly sirolimus metabolism at the hepatic level. However, CYP3A4 strong inhibition by cyclosporine could unveil the influence of this polymorphism.
Sirolimus (previously known as rapamycin) is a potent immunosuppressive drug used following 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) cytochromes P450 (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 due to mutations in the CYP 3A5 gene. Its expression is almost fully abolished in homozygous carriers of the mutated CYP 3A5*3 allele (approx. 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), lower in others (Kamdem et al., 2005). Additionally, there is increasing evidence that CYP 3A4 and CYP 3A5 have a differential susceptibility to enzyme inhibitors (Granfors et al., 2006), so that CYP 3A5 genotype might be a determinant not only of inter-individual 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 CYP3A5 genotypes and dose requirement in patients without calcineurin inhibitor (Anglicheau et al., 2005; Le Meur et al., 2006), but not in patients co-administered 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 (P-gp). This
can lead to pharmacokinetic interactions, as initially described in rats (Stepkowski et al., 1996). In renal transplant patients, co-administration of the two drugs resulted in increased sirolimus AUC (1.45-fold), through concentration (1.49-fold) and maximal concentration (1.72-fold), as compared to 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 CYP3A4-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.

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-Aldrich (St-Louis, MO, USA). Sirolimus was obtained from Wyeth Ayerst (Pearl River, NY, USA), 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 CYP

Pooled human liver microsomes (HLM), microsomes prepared from baculovirus-infected insect cells (Supersomes®) expressing human CYP 3A4 or 3A5 (recombinant CYP) and controls were purchased from BD GENTEST (Woburn, MA, USA). Neither CYP 3A4 nor CYP 3A5 were co-expressed with cytochrome b5.

Human liver microsomes genotyped for CYP 3A5*3

32 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 follow: approx. 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, New-Jersey, USA). A further aliquot (910 µl) of extraction buffer was added in the tube and the mixture incubated for 90 min at 55 ºC. 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 re-suspended in 200 µl of a buffer containing 1mM Na$_2$EDTA and 1 M, pH 7.5 Tris HCl (5:95).

Identification of genotypes

Liver samples were genotyped for the \textit{CYP 3A5*3} single nucleotid polymorphism (SNP) 6986A>G (rs 776746), using Real-time Quantitative Polymerase Chain Reaction (RQ-PCR) based on the 5’ nuclease allelic discrimination assay (ABI PRISM 7000 Sequence Detection System; Applied-Biosystems, Courtaboeuf, France). Primers and probes were as follows: forward primer, CGAATGCTCTACTGTCATTTCTAACCA; reverse primer, TGAAGGGTAATGTGGTCCAAACAG; wild-type probe, TTTGTCTTTCAATATCTC; mutated probe, TTTGTCTTTCAGTATCTC. They were designed on the basis of the sequence of \textit{CYP 3A5} gene using Primer Express 2.0 software (Applied-Biosystems) and purchased from Applied-Biosystems. Briefly, 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 \textit{CYP 3A5*3} allele in the 32 liver specimens was compared to 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 previously described (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 MgCl$_2$, 2 mM NADPH, HLM (0.1 mg protein/ml) or rCYP (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 pre-incubated for 5 min before the reaction was initiated by adding NADPH. Incubations were stopped after 10 minutes with ice-cold acetonitrile (volume/volume). In pilot experiments, control incubations of sirolimus were conducted without microsomes, NADPH or both: sirolimus disappearance was NADPH- and microsomes-dependent. Unless otherwise mentioned, incubation experiments were performed in duplicate.

**Chemical inhibition experiments**

To evaluate the effect of enzyme inactivation by the CYP 3A mechanism-based inhibitor TAO, pooled HLM were pre-incubated 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/l) sirolimus and a further aliquot of NADPH, and stopped after 10 min by 200 µl 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 to those of the control experiment and percent inhibition calculated.

**Enzyme kinetic experiments**

In preliminary experiments, linearity of metabolites formation with increasing microsomal protein concentration (0.1-1 mg/ml), rCYP concentration (10-100 pmol/ml) and incubation time (10-60 minutes) 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 rCYP3A5, 10 min incubation time, and a total volume of 200 µl.

**Determination of sirolimus depletion using HLM, 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 (50µl) were removed at 0, 2, 5, 10, 15, 20, 30 minutes and added to 50 µl of ice-cold acetonitrile to stop the reaction. To allow accurate estimation of sirolimus disappearance even after short incubation time, rCYP 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 to 4.16 µM, i.e. 500 to 5000 µg/l) on sirolimus metabolism by pooled HLM was evaluated following 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 non-linear regression of the log-percentage of cyclosporine concentration versus percentage inhibition (GraphPad Prism, GraphPad Software Inc, San Diego, USA).

Then, the effect of a single cyclosporine concentration (0.62µM, i.e.750 µg/l) approx. half of the IC₅₀ on sirolimus depletion by genotyped HLM, rCYP 3A4 and rCYP 3A5 was evaluated following the incubation procedure described above.

**Analytical methods**

**Sirolimus determination**

Sirolimus determination was performed using turbulent flow chromatography-tandem mass spectrometry system (TFC-MS/MS). The system consisted of a Cohesive 2300 system (Cohesive technologies, Milton Keynes, U.K.) equipped with a CTC HTC PAL autosampler kept at 6°C, 2 binary high-pressure Agilent 1100 pumps, and 3 six-port switching valves controlled by the Aria OS software package. System configuration, parameters and analytical process were previously described in details (Sauvage et al., 2006). Briefly, online extraction was performed at a high flow-rate (1.25 ml/min) on a Cyclone®, 50-µm particle size (50 x 0.5 mm I.D.) column (Cohesive technologies) in alkaline conditions (phase A1: 20 mM
ammonium acetate in water with 0.1 % ammonia; phase B1: mixture of acetone/acetonitrile/propanol-2 [50:30:20; vol/vol/vol]). Chromatographic separation was performed in 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 x 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 TSQ Quantum Discovery MS/MS 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. MS/MS detection was performed in the positive ion, multiple reaction monitoring (MRM) mode following two transitions for sirolimus (m/z 931.3→882.1 m/z; 931.3→864.1) and for the internal standard (IS) 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. Briefly, 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 IS (100 µg/l in acetonitrile). The mixture was vortex-mixed for 15 seconds 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 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 inter-assay 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 analysing incubation medium spiked at 4 concentration levels (1, 5, 25, 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 TFC-MS/MS method described above except that MS 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, metabolites’ 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 $V_{\text{max}}$ calculated according to the Michaelis-Menten model using Winreg 3.1 software (Dr J. Debord, Limoges University Hospital).

The in vitro half-life of total sirolimus depletion ($T_{1/2}$) in HLM, rCYP 3A4 and rCYP 3A5 was calculated using the formula:

$$T_{1/2} = \frac{0.693}{k}$$

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):

$$CL_{\text{int}} = \frac{k}{[\text{microsomal protein or rCYP}]}$$
RESULTS

Sirolimus metabolism by pooled HLM

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, while most other fragment ions were also shifted by +16 or -14 u with respect to those of sirolimus, respectively (Figure 1). The sites of hydroxylation and demethylation were not investigated.

Following our incubation conditions, sirolimus metabolism by pooled HLM was CYP 3A dependent: inactivation of the CYP 3A by TAO resulted in a 99.0 ± 0.8 % and 90.4 ± 2.1 % decrease of hydroxy-sirolimus and demethyl-sirolimus formation rate, respectively, and in a 85.1 ± 17.5 % decrease of sirolimus depletion rate.

During incubation, sirolimus concentrations followed a mono-exponential decay over time (Figure 2), leading to an estimated CL_{int} of sirolimus depletion of 898.3 ± 60.9 µl/mg protein/min (n=3 experiments).

Hydroxy-sirolimus and desmethyl-sirolimus formation rates were correctly fitted by the Michaelis-Menten equation (Figure 2). Kinetic parameters are shown in Table 1. HLM showed a higher affinity and capacity for hydroxy-sirolimus formation, resulting in CL_{int} (V_{max}/K_{m}) approx. twice that of demethylation (Table 1).

The sum of the formation CL_{int} of these two metabolites (96.1 µl/mg protein/min) represented approx. 10 % of the total CL_{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 HLM, with hydroxy-sirolimus and to a lesser extent desmethyl-sirolimus being the main metabolites for both CYPs 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 HLM, hydroxylation of sirolimus by rCYP accounted for a limited part of sirolimus disappearance, with $V_{\text{max}}/K_m$ ratios with rCYP 3A4 and 3A5 representing 10 % and 16 % of the depletion $\text{CL}_{\text{int}}$, respectively (Table 1). The apparent $K_m$ of rCYP 3A4 and rCYP3A5 were close to that observed with pooled HLM (Table 1). Although rCYP 3A5 showed a higher affinity for sirolimus than rCYP 3A4, its $V_{\text{max}}$ was 3-times lower. The resulting $\text{CL}_{\text{int}}$ of hydroxylation ($V_{\text{max}}/K_m$) was approx. 2-fold higher than that of rCYP 3A5 (Table 1). Similarly, the estimated $\text{CL}_{\text{int}}$ of sirolimus depletion was 3.5-fold higher for rCYP 3A4 than 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 CYP3A5*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; 0.195, respectively; p>0.099). The $\text{CL}_{\text{int}}$ of sirolimus depletion and kinetic parameters of sirolimus hydroxylation and demethylation were determined using two pools of microsomes, one comprised 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 $\text{CL}_{\text{int}}$ of sirolimus depletion, hydroxylation or demethylation between the two microsome pools (Table 1 and Table 2). This was statistically confirmed by incubation of sirolimus at 0.11 µM
(100µg/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 CYP3A5*3/*3 genotype, respectively (p=0.42). Metabolites formation rates could not be compared because concentrations were below the LOD of the analytical method in most samples.

**Effect of cyclosporine on the metabolism of sirolimus by human liver microsomes and rCYP3A4 and 3A5**

Cyclosporine inhibited the metabolism of sirolimus (0.11 µM) in pooled HLM: inhibition of sirolimus depletion reached 74.5 ± 5.1 % (n=3 experiments) for 4.16 µM (5000 µg/l) of cyclosporine with a IC₅₀ of 1.08 ± 0.15 µM (Figure 3). Metabolites could hardly be determined after inhibition by cyclosporin. Thus, inhibition of sirolimus hydroxylation or demethylation could not be evaluated accurately.

The effect of cyclosporine at a concentration approx. half of the IC₅₀ (i.e. 0.62 µM) was evaluated on the CLₘₜ of sirolimus depletion by rCYP 3A4, rCYP 3A5 and individual genotyped microsome preparations. Cyclosporine caused a 44 % decrease of the CLₘₜ 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

Following *in vitro* incubation with recombinant CYP3A4 or CYP3A5, as well as human liver microsomes, the major metabolites detected were a hydroxylated and a desmethylated metabolite. This is consistent with the finding of Streit et al. who showed that hydroxy-sirolimus and desmethyl-sirolimus were the main sirolimus metabolites in trough plasma samples from renal transplant patients (Streit et al., 1996). We did not perform formal structural identification, but it can be hypothesised that these two metabolites were 12-hydroxy-sirolimus and 39-O-desmethyl-sirolimus, previously reported as the two main metabolites produced by HLM (Streit et al., 1996). These two metabolites showed low formation rates accounting for approx. 10% of sirolimus depletion by pooled HLM.

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 metabolised. Therefore, sirolimus metabolism was further investigated indirectly, by monitoring substrate loss *versus* time (*in vitro* T1/2 approach). This method involves measurement of substrate consumption at a single low concentration (<Km) and provides an estimate of CLint, which is virtually the sum of all the individual CLint of metabolites’ formation.

Using this method and recombinant CYP3A4 and CYP3A5, CYP 3A4 was found to be a more efficient catalyst of sirolimus than CYP 3A5. This finding was confirmed by a more conventional approach (i.e., kinetic study of metabolites’ formation). Recently, Kamdem et al. as well as Dai et al. performed similar comparisons for tacrolimus and showed that rCYP3A5 was more active than rCYP3A4 (Kamdem et al., 2005; Dai et al., 2006). Differences in experimental conditions cannot account for these opposite findings, because when we compared rCYP 3A5 and 3A4 activities for tacrolimus following the same experimental procedures as for sirolimus herein, we also found a 2.5-fold higher activity of rCYP3A5 compared with rCYP 3A4 (unpublished data). Together with the present results for sirolimus,
this shows that the rCYP 3A5/rCYP 3A4 activity ratio can be totally different depending on
the substrate, even for structurally related molecules such as tacrolimus and sirolimus.

The effect of the CYP3A5*3 polymorphism on sirolimus in vitro metabolism is negligible at
the hepatic level, as neither metabolite production nor sirolimus depletion were significantly
influenced. However, in renal transplant patients, the in vivo clearance of both tacrolimus and
sirolimus is dependent on CYP 3A5 polymorphism: it was demonstrated that patients with the
CYP3A5*3/*3 genotype had significantly lower tacrolimus dose-adjusted through level than
CYP3A5*1 allele carriers (Hesselink et al., 2003; Thervet et al., 2003). Anglicheau et al.
found a significant association between the CYP 3A5*3 SNP and sirolimus dose-adjusted-
trough levels in 69 patients under a sirolimus-based rescue therapy (Anglicheau et al., 2005),
but not in 51 de novo patients nor in 29 patients co-administered cyclosporine or tacrolimus
(Anglicheau et al., 2005). We recently demonstrated that renal transplant patients carrying at
least one CYP 3A5*1 (n=6) allele presented dose-adjusted through level, maximal
concentration and area under the concentration time curve approx. half of those of
CYP3A5*3/*3 patients (n = 41) (p=0.01) (Le Meur et al., 2006). Indeed, the allelic variant
CYP3A5*3 confers low CYP3A5 expression as a result of improper mRNA splicing (Kuehl et
al., 2001; Lin et al., 2002). On the contrary, the presence of a wild-type allele of the CYP3A5
gene (CYP3A5*1) results in high levels of CYP3A5 protein. Thus, CYP 3A5 polymorphism
would obviously have an impact on the first-pass metabolism of both immunosuppressive
drugs. As far as tacrolimus is concerned, the mutation was associated with a decrease in
tacrolimus hepatic and renal metabolism in vitro (Dai et al., 2006). In liver transplant patients,
the genotype of the donor was even found to be a significant determinant of tacrolimus
bioavailability (Goto et al., 2004). In light of 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 adjunct to CYP 3A4, would be of a greater importance for sirolimus presystemic metabolism.

This also suggests that the liver donors’ CYP3A5*3 genotype would be of minor importance for sirolimus pharmacokinetics as compared to that of the recipients.

Cyclosporine was found to inhibit the in vitro metabolism of sirolimus by HLM. 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-gp (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 in 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. about 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 hepatic concentrations). To allow unbiased evaluation of the interaction, we used cyclosporine at a concentration lower that the IC_{50}, and compatible with the concentrations achieved clinically (i.e. 750 µg/l, which is the range of cyclosporine blood concentrations two hours after standard dosing in renal transplant patients).
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 rCYP 3A5. Inhibition was also weaker in HLM derived from expressers of CYP 3A5 compared to non-expressers. It seems that the influence of the CYP 3A5 polymorphism is dependent of CYP 3A4 inhibition by cyclosporine. Differential inhibition of the CYP 3A4 and 3A5 mediated 6-ß-hydroxylation of testosterone (a CYP 3A probe substrate) has already been reported for human immunodeficiency virus protease inhibitors: whereas ritonavir, nelfinavir and saquinavir are non-selective inhibitors of CYP 3A4 and 3A5, indinavir and amprenavir preferentially inhibits CYP 3A4. Gibbs et al. also showed that CYP 3A5 was less susceptible to inhibition by ketoconazole and fluconazole than CYP 3A4 and suggested that this may contribute to inter-individual variability in the magnitude of drug-drug interaction of CYP 3A substrates (Gibbs et al., 1999). Although our results are restricted to the in vitro study of sirolimus metabolism at the hepatic level, it could be hypothesised that the difference in sirolimus exposure between CYP 3A5 expressers and non-expressers could be deeper in the presence of cyclosporine, as the proportion of CYP3A5 metabolism may be increased in the former. However, Anglicheau et al. did not find any gene-exposure relationship in patients co-administered sirolimus and cyclosporine (Anglicheau et al., 2005), 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 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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timing of sirolimus and cyclosporine microemulsion formulation coadministration on


Footnotes

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Legends for figures

Figure 1  Chromatogram of the MRM transitions, and product-ion scans of sirolimus and metabolites (hydroxy-sirolimus and desmethyl-sirolimus) in control incubation and incubation with pooled HLM, respectively.

Figure 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.

Figure 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 1  Kinetic parameters of metabolite formations by rCYP 3A4, rCYP3A5, a commercially available pool of human liver microsomes and two pools of human liver microsomes genotyped for the CYP 3A5*3 single nucleotide polymorphism. Results are expressed as the mean and both value (n=2 experiments) or mean ± SD (n=3 experiments).

<table>
<thead>
<tr>
<th></th>
<th>Hydroxylation of sirolimus</th>
<th>Demethylation of sirolimus</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$(µM)</td>
<td>$V_{max}^a$</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 HLM</td>
<td>2.9 ± 0.5</td>
<td>180.0 ± 9.7</td>
</tr>
<tr>
<td>CYP 3A5*3/*3 HLM</td>
<td>2.0 (2.2; 1.8)</td>
<td>132.5 (134.7; 130.4)</td>
</tr>
<tr>
<td>CYP 3A5*1/*3 HLM</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 CYP/min for rCYP and pmol/mg protein/min for microsomes

$^b$ Units are µl/pmol CYP/min for rCYP and µl/mg protein/min for microsomes

ND: Not Determined (non-saturable metabolite formation)
TABLE 2  Mean intrinsic clearance of sirolimus depletion with and without cyclosporine (and inhibition percentage) in rCYP 3A4 and rCYP3A5 and in two pools of human liver microsomes genotyped for the *CYP 3A5*3 single nucleotide polymorphism. Results are expressed as the mean and both values (n=2 experiments) for CYPr and mean ± SD for microsomes (n=3 experiments).

<table>
<thead>
<tr>
<th></th>
<th>CL_{intrinsic} of sirolimus depletion$^a$</th>
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<tbody>
<tr>
<td></td>
<td>Without cyclosporine</td>
<td>With 0.62µM cyclosporine</td>
<td>Inhibition (%)</td>
<td></td>
</tr>
<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>
<td></td>
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<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>
<td></td>
</tr>
<tr>
<td>Microsomes CYP 3A5*3/*3</td>
<td>1277 ± 63</td>
<td>562 ± 35</td>
<td>52 (56; 59)$^b$</td>
<td></td>
</tr>
<tr>
<td>Microsomes CYP 3A5*1/*3</td>
<td>1265 ± 223</td>
<td>784 ± 166</td>
<td>32 (31; 49)$^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Unit are µl/pmol CYP/min for rCYP and µl/mg protein/min for microsomes

$^b$ Median (min; max)
Figure 1

- **Sirolimus**
  - (-) m/z 931→882
  - (-) m/z 931→864

- **Hydroxy-sirolimus**
  - (-) m/z 947→898
  - (-) m/z 947→880

- **Desmethyl-sirolimus**
  - (-) m/z 917→868
  - (-) m/z 917→850
Figure 2