# Introduction and Overview

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## INTRODUCTION

The purpose of this supplement is to provide the reader with a focused presentation of the results of recent investigations involving sirolimus,* including (1) analytical aspects of the measurement of sirolimus in the blood of renal transplant patients, (2) the pharmacokinetics of sirolimus, and (3) the current state of knowledge regarding the interpretation of the concentration data in relation to measurements of patient outcome.

The authors of the first 6 papers describe, with emphasis on analytical characterization, their experience with sirolimus measurement and sample stability using 1 or more of the following: a high-performance liquid chromatography–ultraviolet (HPLC-UV) method, an experimental microparticle enzyme immunoassay (MEIA),† an experimental sirolimus immunophilin-binding assay, and an HPLC–tandem mass spectrometry (MS/MS) gold-standard reference method. The next 3 papers address the therapeutic drug monitoring of sirolimus, including its pharmacokinetic characteristics, which are the basis for the currently recommended dosing schedule, and the development of a provisional target range for the most effective steady-state blood concentrations. The final paper summarizes ongoing experience with a quality-assessment program that provides feedback to participating centers on the performance of the analytical system used for therapeutic drug monitoring of sirolimus. It is hoped that this information will provide a useful basis for the development of rational therapeutic drug monitoring programs for sirolimus.

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*Trademark: Rapamune® (Wyeth-Ayerst Laboratories, Philadelphia, Pennsylvania).
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## OVERVIEW

### *Mechanism of Action*

Essential to our understanding of the immunosuppression afforded by sirolimus is an understanding of its mechanism of action and interaction with concomitant therapy in the target of immunosuppression, the activated T cell. Multiple molecular and cellular events are important to the complex rejection process. Each immunosuppressant is targeted at primarily one important pathway underlying this process. Sirolimus binds to a member of the immunophilin family of cytosolic binding proteins, FK-binding protein 12 (FKBP12), within the target cells. The sirolimus:FKBP12 complex binds to a specific cell-cycle regulatory protein—mTor (mammalian target of rapamycin)—and inhibits its activation. The inhibition of mTOR results in suppression of cytokine-driven (ie, cytokines such as interleukins [IL] 2, 4, and 15) T-lymphocyte proliferation, inhibiting the progression from $G_1$ to the S phase of the cell cycle (Figure 1).[1–5]

On the other hand, cyclosporine combines with another immunophilin, cyclophilin, in activated lymphocytes. The resulting drug-immunophilin complex inhibits calcineurin, a $Ca^{2+}$/calmodulin-dependent serine/threonine phosphatase required for production of cytokines such as IL-2 and the early activation of T lymphocytes (ie, the transition from the $G_0$ to the $G_1$ phase of the cell cycle).[6] FK-506 combines with FKBP12, but instead of inhibiting mTOR, this drug-binding protein complex inhibits calcineurin, ultimately leading to arrest of proliferating lymphocytes at the transition from the $G_0$ to the $G_1$ phase of the cell cycle by the same mechanism involved in cyclosporine's effects.[7–9]

The mechanism of action of another class of immunosuppressant is that of mycophenolic acid. The latter agent selectively inhibits inosine monophosphate dehydrogenase, resulting in significant reduction of intracellular pools of guanine nucleotide in activated T cells, thereby inhibiting new DNA synthesis followed by the arrest of proliferation at the $G_1$ to the S phase of the cell cycle.[10,11]

Numerous clinical investigations have shown that the most effective prophylaxis of rejection in transplant patients is achieved by combining 2 or more immunosuppressive agents, because no single agent can reduce the risk of acute or chronic rejection to the same degree as can combined therapy.[12,13] In addition, combination therapy with sirolimus and cyclosporine has a synergistic effect that has been demonstrated in in vitro and in vivo preclinical models and in transplant patients.[14–16]

Phase I, II, and III investigations[17–21] in renal transplant patients have demonstrated the efficacy and relative safety of sirolimus, and future investigations should be designed to maximize fully the benefits of this immunosuppressive agent. In addition, sirolimus inhibits proliferation of nonimmune cells (eg, smooth muscle cells stimulated with basic fibroblast growth factor[22] or platelet-derived growth factor[23]). This activity raises the possibility that sirolimus might inhibit the smooth muscle proliferation thought to underlie chronic rejection in transplant patients. Whether this will be the case in human transplantation must await the outcome of investigations of the incidence of chronic rejection in patients receiving long-term therapy with clinically effective doses and blood con-

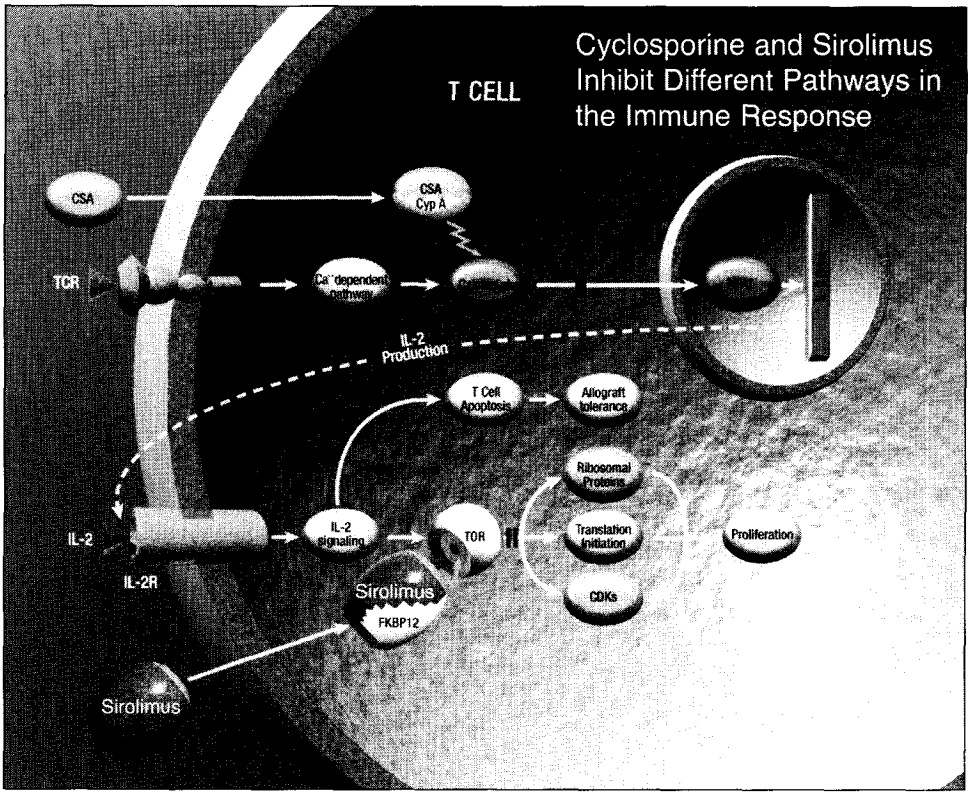


Figure 1. Cyclosporine and sirolimus inhibit distinctly different pathways involved in the immune response.

centrations of sirolimus compared with appropriate controls.

### *Sirolimus Analysis*

The first 6 papers, which were written by individuals with considerable experience in sirolimus analysis, sample handling, and sample stability, describe analytical procedures. The main features of each methodology (Tables I and II) show that important differences between the methods include sample size, sample preparation, method of detection, limit of quantification, estimated time to complete analysis of a batch of blood samples, specificity for sirolimus detection, precision, type of instrumentation and availability in clinical laboratories, and results of studies comparing the investigator's method to a reference method.

It is clear from all the descriptions of sirolimus measurement that, although this is a challenging molecule to measure, available methods meet many or all generally accepted criteria for validated analysis of immunosuppressive drugs. It is also clear that the gold-standard reference method is HPLC–tandem mass spectrometry (HPLC/MS/MS), which we be-

Table I. Primary characteristics of sirolimus analytical methods.

| Characteristic | HPLC-UV I | HPLC-UV II | HPLC-UV III | HPLC/MS/MS | MEIA | IBA |
|---|---|---|---|---|---|---|
| Sample volume (μL) | 1000 | 1000 | 1000 | 500 | 150 | 200–400 |
| Sample preparation | LL × 2 | LL, dry-ice bath | LL, hexane back extraction | PP, solid-phase extraction | PP | Methanol extraction |
| Detection of sirolimus | UV, 278 nm | UV, 278 nm | UV, 278 nm | SRM, m/z 931.8 → 864.6 | MEIA | Tritiated dihydrotacrolimus |
| Calibration range (μg/L) | 2–50 | 2.5–25 | 6.5–356 | 0.2–100 | 3–30 | 2.5–40 |
| LLOQ (μg/L) | 2 | 2.5 | 6.5 | 0.2 | 3 | 2.5 |
| Approximate time to complete 24 samples (h) | 14 | 14 | 12 | 5 | 1.5 | 3 |
| Specificity | Specific for sirolimus* | Specific for sirolimus* | Specific for sirolimus* | Specific for sirolimus | Metabolite cross-reactivity | Weak metabolite cross-reactivity |
| Availability of instrumentation | In many clinical laboratories | In many clinical laboratories | In many clinical laboratories | Not widely available | Not commercially available† | Not commercially available‡ |

HPLC = high-performance liquid chromatography; UV = ultraviolet; HPLC/MS/MS = high-performance liquid chromatography–tandem mass spectrometry; MEIA = microparticle enzyme immunoassay; IBA = immunophilin-binding assay; LL = liquid-liquid extraction; PP = protein precipitation; SRM = selected reaction monitoring abundance ratio match to standard; LLOQ = lower limit of quantification.

*Need to avoid nonspecific interference.

†An investigational immunoassay was developed for sirolimus.

‡Scintillation counter is available in many clinical laboratories.

Table II. Summary of comparison studies for sirolimus analytical methods.*

| Comparison Study | HPLC-UV I vs HPLC/MS/MS | MEIA vs HPLC/MS/MS | MEIA vs HPLC-UV II | MEIA vs HPLC-UV III | IBA vs HPLC-UV I |
|---|---|---|---|---|---|
| Samples | | | | | |
| No. | 385 | 841 | 194 | 133 | 147 |
| Type | From 50 dose-interval profiles of RT patients | Trough samples from 74 RT patients | Trough samples from 194 RT patients | Trough samples from 35 RT patients | (1) 45 dose-interval and (2) 102 trough samples from 86 RT patients |
| Regression slope | 1.068 | 1.37 | 1.10 | 1.27 | (1) 1.09, (2) 0.92 |
| Regression intercept | 0.833 | 1.4 | 0.31 | –1.6 | (1) –0.52, (2) 0.79 |
| Correlation coefficient | 0.95 | 0.96 | 0.94 | 0.90 | (1) 0.98, (2) 0.76 |
| Standard error of estimate | 2.87 | 2.76 | 5.17 | 5.83 | (1) 3.09, (2) 4.10 |
| Average bias | 0.66 μg/L | 42.5% | 10% | 17% | 0.42 μg/L† |

HPLC = high-performance liquid chromatography; UV = ultraviolet; HPLC/MS/MS = high-performance liquid chromatography–tandem mass spectrometry; MEIA = microparticle enzyme immunoassay; IBA = immunophilin-binding assay; RT = renal transplant.

*Analytical methods are those in Table I except for HPLC-UV I versus HPLC/MS/MS, which is the validated method described by Leung et al.[29]

†Trough samples.

lieve will be invaluable in pharmacokinetic-clinical outcome investigations and in sorting out analysis questions for samples with questionable results or suspected interference problems.

Validated HPLC/MS/MS methods were used to estimate sirolimus pharmacokinetic parameters 1, 3, and 6 months after transplant surgery in 42 renal transplant patients enrolled in trial 301,[24] in an investigation involving 40 stable renal transplant patients receiving cyclosporine plus corticosteroid immunosuppressive therapy who received ascending oral doses of sirolimus,[25] and in an investigation of the effect of a fatty meal on sirolimus pharmacokinetics in 22 healthy volunteers.[26]

### *The Experimental Immunoassay*

As described in the paper by MacDonald et al,[24] the investigational microparticle enzyme immunoassay (MEIA) was used for sirolimus quantification in trough blood samples collected in 2 phase III trials (301 and 302) throughout the early posttransplant period. These trough concentration values were used for the retrospective assessment of the relationship between sirolimus concentration and clinical events such as acute rejection and adverse effects.[24] The latter analysis suggests that the target sirolimus trough concentration range is 5 to 15 μg/L with concomitant full-dose cyclosporine therapy.

The MEIA cross-reacts with sirolimus metabolites. Jones et al[27] reported cross-reactivities of a preparation of hydroxy-rapamycin and of 41-O-demethyl-rapamycin of 44% to 50% and 86% to 127%, respectively. Further assessment of the extent of the cross-reactivity for the individual major metabolites, and their actual concentrations in addition to that of sirolimus, is essential for the full characterization of the factors contributing to the bias of the sirolimus immunoassay.

The results of studies comparing the immunoassay with 2 validated HPLC-UV procedures and 1 HPLC/MS/MS method are summarized in Table II. These show that the studies vary in the range of bias (mean bias ranged from 10% to 42%) between the MEIA and the reference HPLC methods. This range could be due to differences in the sirolimus metabolite concentrations and profiles resulting from the use of blood samples obtained from different patient populations at different times within the dose interval or at different times after transplantation.

An interesting observation reported by Salm et al[28] was the significant decrease in the bias in the immunoassay results, which correlated with the steady increase in hemoglobin concentration with time after transplantation (due to recovery of the renal transplant patients from anemia). An earlier observation by Leung et al[29] provides a possible mechanism for this observed change in metabolite bias with improvement in anemia. These authors reported that sirolimus metabolites preferentially distribute into the plasma compartment relative to the parent drug. This suggests that the proportion of metabolites to sirolimus in whole blood will be influenced by anemia, which is often present in renal transplant patients in the early posttransplant period. In the presence of anemia, the proportion of metabolites to sirolimus is presumed to be increased due to the increased proportion of plasma to blood cells, thereby increasing the potential for metabolite bias in whole-blood samples.

Further studies are needed to develop a clearer understanding of (1) the factors that contribute to the metabolite bias of

the immunoassay, (2) the variability of the metabolite bias within and between patients, and (3) the practical significance of these findings for patient management. A multicenter analytical methodology investigation in which participating centers analyze, on multiple days, replicate blood samples including blank samples, a set of sirolimus-spiked samples, metabolite-spiked samples, and pools of blood from transplant patients receiving the drug is recommended. Such a study could go a long way toward characterizing the analytical performance of sirolimus methods and identifying potential sources of error in clinical practice.

### *Sirolimus Metabolism*

The processing of sirolimus by the human body is driven by oxidative metabolism by the CYP3A4 isozyme and the multidrug p-glycoprotein (gp) counter-transporter in the gastrointestinal tract and liver.[30] This processing in the gastrointestinal tract probably accounts for the low (mean, 14%) and variable bioavailability of sirolimus[31] and for the known drug-drug interactions involving sirolimus and cyclosporine, ketoconazole, diltiazem, and rifampin.[24] The parent drug is metabolized to ≥7 metabolites characterized as 41-O- and 7-O-demethyl, several hydroxy, hydroxy-demethylated, and didemethylated sirolimus.[29,30,32]

The structures and metabolic pathways for sirolimus in humans are illustrated in Figure 2. Structural characterization was based on HPLC/MS/MS analyses of metabolite extracts from (1) in vitro studies using human microsomes,[33] (2) trough blood samples in 2 studies in renal transplant patients receiving sirolimus plus cyclosporine and corticosteroid long term,[33,34] (3) blood samples collected in an investigation in healthy volunteers given a single oral 40-mg dose of the $^{14}$C–radiolabeled drug.[29] Seco-sirolimus, a ring-opened derivative of the parent drug, was also detected in the in vitro metabolic studies and in trace quantities in the trough blood samples from renal transplant patients.[29,35]

In the investigation of sirolimus metabolism after administration of the 40-mg dose of $^{14}$C-radiolabeled drug, the percentage of the most prominent metabolites and of the parent drug were estimated using HPLC/MS/MS analysis of extracts of pooled blood samples from 6 healthy volunteers at 3 time points.[29] The unchanged parent drug accounted for 67%, 45%, and 51% of the total of all detected radioactive sirolimus-derived products at 2, 12, and 24 hours, respectively, after drug administration. Two other investigations assessed the relative proportion of sirolimus and metabolites in trough whole-blood samples obtained from stable renal transplant patients receiving concomitant cyclosporine and corticosteroid therapy.[33,34] The relative abundance of unchanged sirolimus and metabolites were (in decreasing order) sirolimus>hydroxy metabolites>demethylated metabolites> hydroxy-demethyl>di-demethyl. Total sirolimus metabolites accounted for 48% to 70% in 1 investigation[34] and an average of 56% in another[32] of recovered sirolimus derivatives. According to 1 report, no single metabolite was present at concentrations >10% that of sirolimus in trough whole-blood samples from 10 stable renal transplant patients.[34]

Given the scarcity of purified metabolites and the difficulty of isolating each to purity, there has been limited opportunity to assess their biologic activity fully. In 2

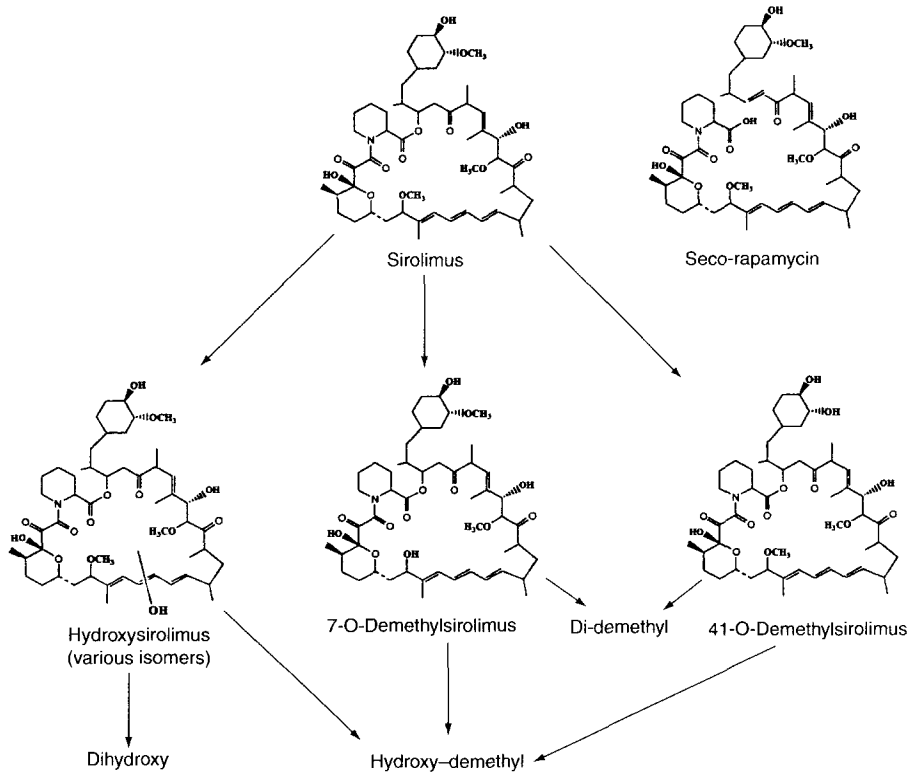


Figure 2. The metabolic pathways for sirolimus in human beings.

investigations of sirolimus metabolites or metabolite mixtures,[29,36] immunosuppressive activity was <10% to 30% of the in vitro immunosuppressive activity of the parent drug in lymphocyte and thymocyte proliferation assays. Given the reported relatively low concentration of any single metabolite in renal transplant patients' blood, it is unlikely that metabolites contribute significantly to the overall immunosuppressive activity of sirolimus.[30]

### *Sirolimus Pharmacokinetics*

In an ascending multiple-oral-dose pharmacokinetic study of 40 stable renal transplant patients receiving concomitant cyclosporine and corticosteroid therapy, the elimination of sirolimus was slow, as reflected by a half-life of 62.3 ± 16.2 hours.[25] Based on this half-life, 2 important recommendations for sirolimus administration in renal transplant patients are (1) once-daily dosing and (2) use of a single loading dose 3 times the maintenance dose when immunosuppression is initiated in de novo transplant patients. Both recommendations were used in trials 301 and 302. Consistent with this long half-life was an earlier observation of an average 2.5-fold accumulation of sirolimus concentration, measured in trough

Table III. Pharmacokinetic parameters for sirolimus in 42 de novo adult renal transplant patients.*

| Sirolimus Dose (mg/d) | $C_{max}$† (μg/L) | $t_{max}$ (h) | AUC† (μg*h/L) | $C_{min}$† (μg/L) | Cl/F (mL/h/kg) |
|---|---|---|---|---|---|
| 2 (n = 19) | 12.2 ± 6.2 (51.1) | 3.1 ± 2.4 (79.6) | 158 ± 70 (44.1) | 4.6 ± 2.3 (50.6) | 182 ± 72 (39.7) |
| 5 (n = 23) | 37.4 ± 21.0 (56.2) | 1.8 ± 1.3 (70.4) | 396 ± 193 (48.7) | 10.8 ± 5.4 (49.9) | 221 ± 143 (64.7) |

$C_{max}$ = peak whole-blood sirolimus concentration; $t_{max}$ = time to peak concentration; AUC = area under the curve. $C_{min}$ = trough concentration; Cl/F = oral dose clearance.

*Each result is the average value ± SD from determinations made at the end of months 1, 3, and 6 using a validated HPLC/MS/MS method. The numbers in parentheses are the percentage coefficient of variation for interpatient variability. These pharmacokinetic data were obtained for patients enrolled in trial 301.

†These parameters were normalized to the standard dose used in the respective treatment group.

whole-blood samples, over ~6 days of fixed-dose administration. Thus, it takes ~6 days of fixed-dose administration to reach a steady-state concentration.

The analytical method used in the ascending multiple-dose study was a validated HPLC/MS/MS procedure, in which the ratio of the intensities of the positive sodium ion adducts of sirolimus and the internal standard, 32-desmethoxyrapamycin, mass-to-charge ratio 937 and 907, respectively, were used to calculate the concentration of sirolimus with a calibration curve.[25] Another outcome of interest in that investigation, in which study patients received 2 daily doses of sirolimus 3 hours after their cyclosporine dose, was the observation that the trough concentrations, obtained at the end of the 12-hour dose interval, correlated well with the 12-hour dose interval area under the curve (AUC) ($r^2$ = .94). However, the predose trough correlation was not as good but was still significant ($r^2$ = .85). This suggests that the predose trough value may be a reasonably good predictor of the sirolimus dose-interval AUC. Further investigations of this relationship are recommended, because this well-done study used a twice-daily dosing regimen rather than the now-recommended once-daily schedule. Furthermore, there is likely to be more variability in the measured predose trough concentration under routine clinical practice conditions because of uncertainties in the actual time between the last dose and the time the blood sample was obtained and other factors, such as possible recent meal consumption.

Table III summarizes the primary pharmacokinetic parameters obtained at the 2 daily doses used in trial 301. Statistical analysis of these data using analysis of variance showed no significant difference for any of the parameters for race, time after transplantation, or treatment group (sirolimus 2 or 5 mg/d). Because interpatient variability was high for all pharmacokinetic parameters (as indicated by the large percentage of coefficient of variation values), caution is needed in interpreting these data. Only very large differences would be detected. Further investigations using sufficiently large numbers of patients are required to assess the possible

Table IV. Provisional therapeutic drug monitoring strategy for sirolimus.

| | |
|---|---|
| Analytical method: | Validated HPLC-UV or HPLC/MS/MS |
| Sample: | Whole blood |
| Sample timing: | Predose, 24-hour trough, abbreviated AUC* |
| Proposed monitoring schedule: | After the loading dose to ensure values are not at the extremes of the percentile distribution (eg, <5 ng/mL on 2 consecutive determinations within the first 2 weeks after transplantation) |
| | After new steady state is reached (~5 to 7 days after a dose change) if a dose change was made within the first 2 weeks after transplantation |
| | In the patient at average risk |
| | After introduction or discontinuation of strong inhibitors or inducers of CYP3A4 or P-glycoprotein transporter |
| | After changes in cyclosporine dosing and steady-state blood concentrations |
| | When there is a significant change in the relative timing of sirolimus and cyclosporine doses |
| | When there are significant changes in the patient's condition (eg, development of liver disease, hyperlipidemia, thrombocytopenia, leukopenia) |
| | To check compliance |
| | Closer monitoring of sirolimus blood concentrations is recommended in pediatric patients, patients at high risk for rejection, and patients with hepatic impairment |

HPLC = high-performance liquid chromatography; UV = ultraviolet; MS = mass spectrometry; AUC = area under the curve.

*Further investigations are recommended to assess the cost-benefit ratio of an abbreviated sirolimus AUC compared with 24-hour trough sample time.

sources of variability in the pharmacokinetic parameters.

Considerable variability in sirolimus pharmacokinetic parameters was expected based on the known variability in enterocyte CYP3A4 content (9.4-fold in 25 renal transplant patients[37]) and enterocyte p-gp content (8.5-fold). The wide variability in CYP3A4 processing of sirolimus was recently confirmed in an investigation of sirolimus metabolism using microsomal preparations obtained from duodenal tissue from 14 patients. This investigation showed an 8-fold range in the rate of production of sirolimus metabolites in the test system.[30]

### *Therapeutic Drug Monitoring*

Results of investigations of sirolimus in animal transplant models suggest that whole-blood trough concentrations of siro-

limus of ~5 to 10 μg/L provide efficacy.[38] Whole-blood trough concentrations of sirolimus were determined throughout the 6-month study period in trials 301 and 302 using MEIA in its original configuration (Mode 1A calibration).[24] The renal transplant patients enrolled in these 2 trials received concomitant full-dose cyclosporine guided by whole-blood concentration monitoring and corticosteroid therapy. Dosing of sirolimus was fixed at 2 or 5 mg/d and not guided by therapeutic drug monitoring in these trials. The major findings of the analyses of the study data are as follows[24]:

From trials 301 and 302, several issues become apparent regarding the relationship between sirolimus whole-blood trough concentrations and risk for rejection or adverse effects.[24] The wide inter- and intrapatient variability of sirolimus trough concentration versus dose makes it difficult to predict the trough concentration from the dose. However, when sirolimus is given with full-dose cyclosporine, this variability is such that for the patient at average risk (ie, not pediatric patients or those at high immunologic risk), there appears to be less need for therapeutic monitoring unless patients had significant changes in their immunosuppressive regimen, were taking other medications, their compliance was suspect, or their health status warranted monitoring (Table IV).

## CONCLUSIONS

Therapeutic drug monitoring of sirolimus is in its infancy. Further experience will be gained through its use in clinical practice, and investigations will undoubtedly assess the risk/benefit ratio for therapeutic drug monitoring using improved methods for analysis of blood concentrations. Based on such studies, dosing of sirolimus in individual patients will undoubtedly be refined. To maximize this drug's potential benefits, a number of investigations will be of great interest, including its use with other agents (eg, tacrolimus) at conventional or reduced dose and blood concentrations. This also will be true for the combination of low-dose cyclosporine and sirolimus. In each of these instances, assessment of the most-effective sirolimus blood concentrations in the presence of lower calcineurin inhibitor will be important. These are just 2 of a number of possible new immunosuppressive regimens.

For the full-dose cyclosporine plus sirolimus combination, we believe a reasonable therapeutic drug monitoring approach would include an early determination, after the loading dose, of ≥2 trough concentrations to ensure that the patient's value is not at an extreme of the percentile distribution. Thus, for example, if 2 consecutive trough concentrations were <5 mg/L during the first 2 weeks after administration of the loading dose, a dose increase would be advisable to ensure adequate sirolimus exposure.

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