Longitudinal assessment of everolimus in de novo renal transplant recipients over the first post-transplant year: Pharmacokinetics, exposure-response relationships, and influence on cyclosporine

Objective: **Our objective was to characterize the steady-state pharmacokinetics of everolimus and cyclosporine (INN, ciclosporin) when coadministered in de novo kidney allograft recipients during the first year after transplantation.**

Methods: **This study was a multicenter randomized double-blind study of 101 patients who were randomly assigned 1:1:1 to receive everolimus tablets at doses of 0.5 mg, 1 mg, or 2 mg twice daily with cyclosporine and prednisone. Blood sampling for the pharmacokinetics of everolimus and cyclosporine was performed on day 1, on weeks 1, 2, 3, and 4, and on months 2, 3, 6, 9, and 12. Everolimus dose-proportionality and stability over time were assessed in the context of linear regression and ANOVA models. Everolimus exposure-response relationships between area under the blood concentration-time curve (AUC) and changes in platelets, leukocytes, and lipids were explored with the median-effect model. Potential differences in cyclosporine dosing and pharmacokinetics at different levels of everolimus exposure were assessed in the context of ANOVA.**

Results: **Everolimus steady state was reached on or before day 7, with a median 3-fold accumulation of drug exposure compared with that after the first postoperative dose. Both steady-state maximum concentration and AUC were dose proportional over the full dose range when assessed on day 1, as well as for the full duration of the study at steady state. There was evidence for longitudinal stability in AUC of everolimus during the course of the study. The interindividual pharmacokinetic variability for AUC was 85.4% and intraindividual, interoccasion variability was 40.8%. Age (range, 17-69 years), weight (range, 49-106 kg), and sex (65 men and 36 women) were not significant contributors to variability. There was an increasing incidence of transient thrombocytopenia ($\leq 100 \times 10^9$/L) with increasing everolimus AUC ($P = .03$). Cyclosporine doses, trough concentrations, and AUC exhibited similar temporal patterns during the course of the study regardless of the co-administered everolimus dose level ($P = .13$, .82, and .76, respectively).**

Conclusions: **Everolimus exhibited dose-proportional, stable exposure during the first post-transplant year. For a 4-fold range of everolimus doses there were no differential effects on cyclosporine dosing or pharmacokinetics. (Clin Pharmacol Ther 2001;69:48-56.)**

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Supported by research grants from the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK#38016-12) and Novartis Pharmaceuticals.

Received for publication Aug 9, 2000; accepted Nov 14, 2000.

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0009-9236/2001/$35.00 + 0 **13/1/112969**

doi:10.1067/mcp.2001.112969

Par Pharm., Inc.
Exhibit 1050
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Case IPR2016-00084

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Temporal changes in the pharmacokinetics of immunosuppressants used in acute rejection prophylaxis after organ transplantation have been reported.[1-4] Two examples are cyclosporine (INN, ciclosporin) and mycophenolate mofetil. For the non-microemulsion formulation of cyclosporine the relationship between dose and area under the blood concentration-time curve (AUC) changes in the first 3 to 4 months after a transplant and stabilizes thereafter.[1,2] Although this pattern is also evident for the microemulsion formulation, the period of dose-AUC stability is achieved earlier (by the end of the first month) compared with the non-microemulsion formulation.[3] This suggests that the oral absorption and bioavailability of cyclosporine in the early post-transplant period (which is improved with the microemulsion formulation) is probably contributing to this phenomenon. For mycophenolate mofetil, dose-normalized AUC is lower in the early post-transplant period and increases slowly over several months by an average 50% to reach late post-transplant values. Because bioavailability is nearly complete throughout this period, poor absorption cannot explain these observations. Speculation has centered on changes in protein binding and enterohepatic recirculation.[4] These examples underscore the importance of characterizing the longitudinal pharmacokinetics of new immunosuppressants, especially in the early post-transplant period so that time-dependent changes in the disposition of the immunosuppressants may be taken into account in the dosing regimen to yield more stable systemic exposure over time.

Everolimus (development name, RAD) is a macrocyclic lactone immunosuppressant that is primarily metabolized and eliminated in the bile. At therapeutic concentrations, more than 75% of everolimus is partitioned into red blood cells and approximately 75% of the plasma fraction is protein bound. Everolimus blocks growth factor–driven transduction signals in the T-cell response to alloantigen[5] and thus acts at a later stage than the calcineurin inhibitors cyclosporine and tacrolimus. The complimentary modes of action of everolimus and cyclosporine suggest a synergistic interaction that has indeed been shown in vitro and in vivo in preclinical models.[5] This provided a rationale for the addition of everolimus to cyclosporine-based immunosuppression. Subsequent phase 1 studies showed good tolerability and an acceptable side-effect profile when everolimus was administered for 1 month to stable patients with renal transplants who were receiving a cyclosporine-prednisone regimen.[6,7] A recently completed phase 2 study constituted the first experience with everolimus in patients immediately after kidney transplantation in the de novo setting.[8] In that trial, extensive pharmacokinetic evaluations were performed during a 1-year period after transplantation for longitudinal characterization of the disposition of everolimus. The clinical results will be reported elsewhere; this article focuses on the pharmacokinetic results of the study in which dose proportionality, longitudinal stability in exposure, and pharmacokinetic variability with potential covariates were addressed.

The inhibitory effects of everolimus are not restricted to T cells. Everolumus also inhibits the signals provided by some hematopoietic and nonhematopoietic cell growth factors.[5] In this context, phase 1 experience with this compound in patients indicated that treatment with everolimus may be associated with decreases in platelets and leukocytes and increases in lipids that generally occur in the first 2 months after initiation of treatment.[6,7] This has also been the experience with sirolimus.[9,10] Because we used a broader range of everolimus doses in this phase 2 study than are currently under investigation in phase 3 efficacy trials, it provided the opportunity for us to screen for exposure-response relationships between AUC and changes in laboratory parameters.

Both everolimus and cyclosporine are extensively biotransformed by means of CYP3A and are substrates for P-glycoprotein. Therefore there is a potential for a drug-drug interaction when these two agents are coadministered. Although everolimus was the primary focus of this study, the longitudinal influence of everolimus on cyclosporine pharmacokinetics was also explored as a secondary objective.

METHODS

Study design and dose regimens. This was a 1-year, randomized, double-blind trial performed at 8 study centers. The study protocol was approved by local medical ethics committees for each center. One hundred three de novo kidney allograft recipients gave written informed consent to participate in the study and were randomly assigned to receive everolimus (Certican, Novartis Pharmaceuticals, East Hanover, NJ) at oral doses of 0.5 mg (n = 34), 1 mg (n = 34), or 2 mg (n = 35) twice daily in addition to cyclosporine and prednisone. The first dose of everolimus was given once it had been ascertained that the allograft was functional (maximum, 48 hours after transplantation). This was defined as study day 1. Everolimus was supplied as a tablet formulation in strengths of 0.25 mg and 1 mg with matching placebos. Patients were instructed to take the prescribed number of tablets every 12 hours simultaneously with cyclosporine. Cyclosporine (Neoral, Novartis Pharmaceuticals) was initiated orally at 6 to 12

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mg/kg/d in two divided doses. Thereafter, the doses were adjusted to maintain whole blood predose concentrations in the range of 150 to 400 ng/mL in the first post-transplant month and in the range of 5 to 300 ng/mL from month 2 to month 12. Prednisone was dosed according to a protocol-specified taper.

Pharmacokinetic assessments. Blood sampling for the pharmacokinetics of everolimus and cyclosporine was performed at protocol-scheduled visits throughout the study duration: day 1, weeks 1, 2, 3, and 4, and months 2, 3, 6, 9, and 12. At each visit, either a full pharmacokinetic profile that consisted of 10 blood samples (predose and 0.5, 1, 1.5, 2, 3, 4, 6, 9, and 12 hours) or an abbreviated profile (predose, and 1, 2, 5, and 8 hours or predose, and 1, 2, and 5 hours) was performed during the morning dosing interval. At the week 3 and month 2 and 12 visits, only a predose trough sample was obtained. For the 5-point abbreviated profiles, the 12-hour concentration was estimated by log-linear decrease from the 5-hour and 8-hour measured concentrations; for the 4-point abbreviated profiles, the 12-hour concentration was assumed to be the same as the predose measured concentration under the assumption of steady state. When maximum concentration (C_{max}) and AUC from the full profiles in this study were re-evaluated on the basis of the 4- or 5-sample abbreviated approach described previously, they were highly correlated ($r^2 = 0.91$ for C_{max} and $r^2 \geq 0.96$ for AUC); this indicated that parameters from full and abbreviated sampling could be pooled for analysis.

Bioanalysis of everolimus. Everolimus whole-blood concentrations were determined with a validated enzyme-linked immunosorbent assay. Performance was assessed on the basis of a 5-point quality control concentration range from 2 to 80 ng/mL of everolimus. Coefficients of variation ranged from 13.3% to 16.1% and bias ranged from –7.0% to –1.8%. The assay quantification limit was 2 ng/mL.

Sample preparation consisted of the mixing of 250 µL of blood sample with 1 mL of extraction buffer (Tris buffer, 0.05 mol/L, pH 9) and 1 mL of extraction solvent (diethyl ether with 0.04% Tween 20). The samples were shaken for 10 minutes and centrifuged for 10 minutes at 4000 rpm and 4°C. They were subsequently frozen in dry ice, and the organic phase was decanted and evaporated at 40°C for 20 to 30 minutes. The dry residues were reconstituted with 120 µL of a reconstitution solution that consisted of 200 ng/mL mouse antirapamycin antibody in phosphate-buffered saline solution (PBS) buffer (1:10 dilution) with 1% bovine serum albumin. For the determination of nonspecific binding, the dry residue was dissolved in dilution buffer without antibody. The samples were shaken for 10 minutes and then incubated for at least 15 minutes at room temperature.

Plastic 96-well assay plates were coated overnight with 120 µL per well of 10 µg/mL goat anti-mouse antibody in PBS buffer (Goat Anti-Mouse IgG Fc, Immunopure, Rockford, Ill) at a temperature of approximately 4°C. The plates were then washed with washing buffer (PBS buffer with 0.05% Tween 20) and blocked with 300 µL per well of blocking solution by incubation for 5 minutes at room temperature. The plates were washed and 100 µL per well of the processed blood samples was added. After 1 hour of incubation at room temperature with gentle shaking, 20 µL of tracer solution (19 ng/mL of rapamycin biotinyled in PBS buffer with 1% bovine serum albumin and 0.04% Tween 20) were added to each well. The plates were incubated overnight at approximately 4°C and then washed with PBS buffer with 0.05% Tween 20 and incubated with 120 µL per well of streptavidin peroxidase solution (diluted 1:10,000 in assay buffer) for 15 minutes in the dark at room temperature with gentle shaking. Plates were washed with assay buffer and 120 µL per well of o-phenylenediamine dihydrochloride solution (10 mg in 50 mL of 0.05 mol/L citrate/phosphate buffer and 20 µL of hydrogen peroxide 30%) was added, and then the plates were shaken gently for 5 to 6 minutes at room temperature. The reaction was stopped with 50 µL per well of 2 N sulfuric acid, and the optical density was measured at 492/620 nm.

Bioanalysis of cyclosporine. Cyclosporine whole blood concentrations were determined with the use of the Incstar CYCLO-Trac radioimmunoassay kit (Stillwater, Minn) according to the manufacturer's directions. Performance was assessed on the basis of a 5-point quality control concentration range from 30 to 1250 ng/mL. Coefficients of variation ranged from 5.1% to 10.6% and bias ranged from –12.0% to 0.4%. The assay quantification limit was 30 ng/mL.

Pharmacokinetic and statistical evaluation. Conventional noncompartmental steady-state pharmacokinetic parameters were derived for both everolimus and cyclosporine, including the predose trough concentration (C_{min}); the C_{max}; the time to reach maximum concentration (t_{max}); the AUC during a dosing interval, by trapezoidal summation; the average concentration (C_{avg}), calculated as the quotient of AUC/12; and the peak-trough fluctuation, calculated as $(C_{max} - C_{min})/C_{avg}$. The accumulation ratio was derived from the ratio of AUCs from day 7 and day 1.

Dose proportionality for the C_{max} and AUC of everolimus was assessed on day 1 (first dose) and day 7 (steady state) with linear regression analysis of the

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Table I. Everolimus pharmacokinetic parameters*

	0.5 mg bid	*1 mg bid*	*2 mg bid*
First dose			
t_{max} (h)	3 (1-12)	3 (2-9)	3 (2-12)
C_{max} (ng/mL)	2.0 ± 2.1	5.6 ± 3.7	9.8 ± 7.0
AUC (ng · h/mL)	8 ± 12	28 ± 23	56 ± 37
Steady state†			
C_{min} (ng/mL)	1.5 ± 1.8	4.7 ± 2.6	9.5 ± 5.2
t_{max} (h)	2 (1-5)	2 (1-5)	2 (1-8)
C_{max} (ng/mL)	5.0 ± 2.9	11.6 ± 4.4	21.9 ± 10.5
C_{max}/dose (ng/mL/mg)	10.0 ± 5.8	11.6 ± 4.4	11.0 ± 5.3
AUC (ng · h/mL)	34 ± 23	81 ± 34	164 ± 78
AUC/dose (ng · h/mL/mg)	68 ± 46	81 ± 34	82 ± 39
C_{avg} (ng/mL)	2.8 ± 1.9	6.7 ± 2.8	13.6 ± 6.5
PTF (%)	84 ± 38	97 ± 38	90 ± 47

bid, Twice a day; PTF, peak-trough fluctuation.
*Values are median (range) for t_{max} and mean ± standard deviation for all other parameters.
†Steady-state data are from week 1.

parameter versus dose. Steady-state, dose-normalized parameters from day 7 to the end of the study were also evaluated in an ANOVA model with dose, subject-within-dose, visit, and dose-by-visit interaction terms. In the absence of a dose-by-visit interaction, lack of a dose-effect was taken as support for dose proportionality during the full study course; lack of a visit effect was evidence for longitudinal stability in exposure. Steady-state AUC per dose from the replicate pharmacokinetic profiles was assessed with a two-way ANOVA with subject and visit as sources of variation. The mean square from the subject term was taken as a measure of interindividual variance, and the mean square from the error term was taken as a measure of intraindividual, interoccasion variance. The corresponding coefficients of variation were calculated as the standard deviation (square root of the mean square) divided by the grand mean of the parameter. The contribution of conventional demographic covariates to the pharmacokinetic variability was explored with graphical and regression techniques for continuous variables (age and weight) and with unpaired two-sided *t* tests for categorical variables (sex and ethnicity).

Dose-normalized cyclosporine pharmacokinetic parameters were evaluated with a global ANOVA model identical to that for everolimus in which the dose term referred to the everolimus dose level. The absence of a dose-by-visit interaction and of a dose-effect was taken as support that the influence of everolimus on cyclosporine was not different among the everolimus dose levels.

Exposure-response relationships. Relationships between everolimus steady-state AUC and the incidence of thrombocytopenia (≤100 × 10^9/L), leukopenia (≤4.0 × 10^9/L), hypertriglyceridemia (>2.9 mmol/L), and hypercholesterolemia (>6.5 mmol/L) were explored with the median-effect principle.[11] This model relates the fraction of the population affected (fa) and unaffected (fu = 1 – fa) with respect to a given laboratory parameter change on the one hand and to the drug exposure (AUC) and the exposure at which half the population is affected (median effect [AUCm]) on the other hand: $fa/fu = (AUC/AUCm)^m$. The relationship is linearized on the logarithmic scale as follows: log(fa/fu) = m log(AUC) – m log(AUCm). In this relationship, m is a Hill-type coefficient that describes the sigmoidicity in the exposure-response relationship. The average AUC for each patient was determined from the week 1, 2, and 4 profiles and the overall distribution divided into quartiles. The fraction of patients whose laboratory parameter did (fa) and did not (fu) exceed the defined cutoff value listed previously in the first two post-transplant months was determined in each of the quartiles. The resulting log(fa/fu) versus log(AUC) relationship was assessed by linear regression analysis. Goodness of fit of the model to the data was indicated by a regression coefficient (*r* value) of more than 0.8; a regression *P* value of less than .05 indicated a significant relationship between exposure and the occurrence of a laboratory parameter change.

RESULTS

Demographics and patient disposition. Of the 103 patients enrolled in the study, 101 provided at least one pharmacokinetic profile and were included in this analysis. The two unevaluable patients withdrew from the trial in the first week and did not undergo pharmacokinetic

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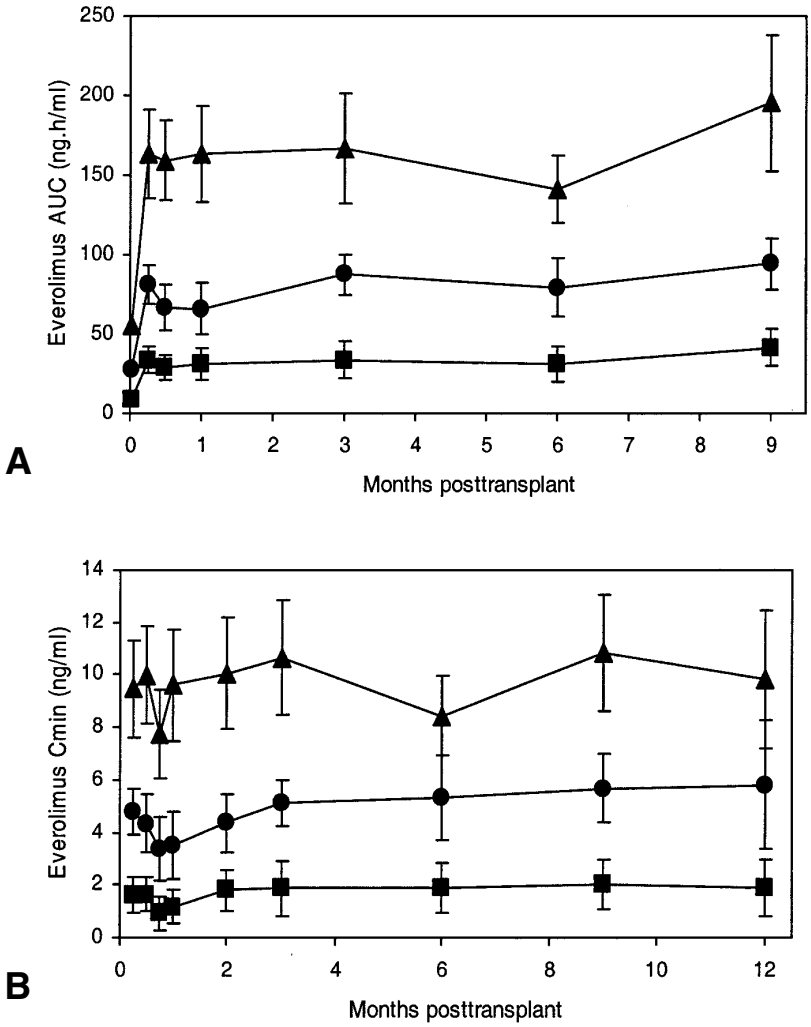

Fig 1. Everolimus mean AUC trajectories during 9 months after transplants **(A)** and everolimus trough concentrations (C_{min}) to 12 months after transplants **(B)** at dose levels of 0.5 mg *(solid squares)*, 1 mg *(solid circles)*, and 2 mg twice a day *(solid triangles)*. *Bars* designate 95% confidence intervals.

blood sampling. There were 65 men and 36 women who were 44.4 ± 11.6 years old and who weighed 76.7 ± 15.4 kg. The majority of the patients were white (n = 82); there were 9 black patients, 1 Asian patient, and 9 patients of other ethnicities.

Everolimus pharmacokinetic profiles were evaluable at each visit if the patient remained on the dose to which he or she was randomly assigned and had not missed doses within 5 days before the visit. Over the 1-year duration of the study, 18 patients had dose reductions for safety reasons, 31 patients had transient dose interruptions, and 16 patients were removed from the study (8 unsatisfactory therapeutic response, 3 died, 3 withdrew consent, and 2 violated the study protocol). Consequently, the number of evaluable profiles at each of the steady-state visits were: 94 (week 1), 88 (week 2), 83 (week 4), 71 (month 3), 58 (month 6), and 55 (month 9).

First-dose everolimus pharmacokinetics. A total of 95 profiles were obtained after the first dose of everolimus on day 1: 33, 32, and 30 profiles, respectively, were obtained from patients who were receiving 0.5, 1, and 2 mg twice daily. First-dose pharmacokinetic parameters are summarized in Table I. In postoperative conditions, there was generally a 1-hour lag time until everolimus was quantifiable in blood, and the peak concentration occurred at 3 hours after the dose. Both C_{max}

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