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Impact of Single Immunosuppressive Drug Withdrawal on Lymphocyte Immuno-reactivity

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Abstract

Chronic rejection is a major cause of graft loss in kidney transplant recipients. Non-adherence to drug therapy is a well-recognized cause of chronic rejection leading to long-term graft dysfunction and failure for transplant recipients. Immunosuppressive medications with short half-lives that require frequent dosing, such as tacrolimus, complicate transplant regimens and may increase noncompliance. Regimens could be simplified using drugs with long half-lives requiring once daily administration, such as sirolimus. The impact of missing doses of single agents has not been studied extensively. Erratic compliance or temporary discontinuation of immunosuppressive drugs may have significant implications for chronic rejection.

Our study evaluated the impact of single drug withdrawal of commonly used immunosuppressive agents (sirolimus and tacrolimus) on lymphocyte responses. We analyzed lymphocyte proliferation, cytokine secretion, and ATP generation using a crossover study design with normal healthy subjects. Lymphocyte proliferation was assessed utilizing BrdU incorporation and T cell function was analyzed by examining ATP generation.

Our results indicate that sirolimus exerts prolonged suppression of lymphocyte proliferation as well as decreased IL-17A that lasts up to 48 hours after drug withdrawal. In comparison, tacrolimus did not have a similar effect on lymphocyte proliferation or IL-17A secretion. Future analysis of sirolimus in diverse transplantation populations merits investigation.

Introduction

Non-adherence with immunosuppressive drug regimens remains a major problem in the long-term management of transplant patients. Presently, the most common regimens include a calcineurin inhibitor (CNI), such as cyclosporine or tacrolimus, which blocks calcineurin signal transduction and prevents activation of the transcription factor nuclear factor of activated T cells (NFAT). Alternative agents that block the mTor pathway, including

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sirolimus, are available but used less commonly due to repression of wound healing and dyslipidemia. CNIs have a shorter half-life (approximately 16–27 hours for cyclosporine in patients with normal liver function(1) and 12 hours for tacrolimus(2)) than sirolimus and are dosed twice daily. In contrast, sirolimus has a longer half-life (62 hours(3)) and is dosed once a day. Thus, the effective management of CNIs requires frequent monitoring of serum drug concentrations and patient adherence.

A recent cross-sectional study of transplant recipients concluded that there is significantly lower graft survival in patients that are assessed to be non-adherent to transplant medication regimens. As expected, the study also revealed higher late acute rejection rates in this group(4). A prospective trial indicated that 47% of transplant recipients with acute rejection are identified as non-adherent(5).

Sommerer and colleagues have shown a close relationship between CNI concentrations (cyclosporine or tacrolimus) over the dosing cycle and cytokine transcription, which could promote the development of graft rejection(3). An analysis of pediatric patients (age 8–18 yrs) showed that patients who received a heart, lung, or kidney transplant and had a higher variability in tacrolimus concentrations were at an increased risk of graft loss and late rejection(6). One interpretation is that non-adherence may be a contributing factors to the variability in tacrolimus concentrations.

We utilized a crossover study design of normal healthy subjects treated with a single-drug regimen of tacrolimus or sirolimus and analyzed drug concentration, lymphocyte proliferation, cytokine secretion, and ATP generation. In this study, we tested the hypothesis that sirolimus will have greater drug concentrations over the dosing interval and maintain greater immunosuppressive effects. We also postulated that these effects are sustained. Consequently, if a dose of sirolimus is taken irregularly or intermittently, this may be less likely to result in an enhanced immune response that promotes graft rejection. Recently, IL-17A has been implicated to have a role in graft rejection(7). Th17 cells are the most recently identified member of the $CD4^+$ T cell subset and secrete IL-17A(8). There are several isoforms of IL-17 that include IL-17A to IL-17F, and they are homodimeric peptides between 35 and 52 kD(9). In this study, we analyzed the impact of withdrawal of sirolimus and tacrolimus on immune reactivity and levels of IL-17A.

Materials and Methods

Subjects

Subjects were recruited from the general population in San Diego (n=5) in accordance with the University of California, San Diego Human Research Protection Program. Inclusion criteria for study subjects was age 18–65 years, body mass index 30 or less, and normotensive with no current or recent infections. Exclusion criteria included a history of prior or existing health problems, such as diabetes, hypertension, heart disease, kidney disease, recurrent infections, neoplasm (or history of such), chronic obstructive pulmonary disease (COPD), peripheral vascular disease (no palpable foot pulses), and/or prescription or over the counter medications except for occasional acetaminophen or non-steroidal anti-inflammatory drugs. A signed informed consent form was obtained from each participant

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prior to any study procedures. Subjects with proteinuria greater than 300 gm/dl, fasting cholesterol greater than 300 mg/dl, fasting triglycerides greater than 400 mg/dl, platelets less than 100,000/mm^3, and/or white blood cell count less than 2.5 mm^3 were excluded.

After the initial recruitment phase, a complete chemistry panel, including liver function tests, complete peripheral blood count with manual normal white cell differential, lipid panel, HgbA1c, and urinalysis were obtained to rule out any occult abnormalities. Pregnancy tests were done for female subjects to rule out pregnancy and subjects were counseled to use an effective form of contraception during the entire trial through the 30-day safety follow up visit.

After recruitment, subjects underwent a baseline 2 hour oral glucose tolerance test (OGTT) and received a single test dose administration of the assigned medication, tacrolimus or sirolimus, to determine optimal dose.

Pharmokinetics Models

Population and individual patient pharmacokinetic modeling was performed using NONMEM (version 6, Icon US, Ellicott City, MD). A two-compartment model (subroutine advan4 trans1) with first-order absorption pharmacokinetics were calculated using the tacrolimus dose and the blood concentration values (blood concentration at t = 0, 2, 12, 24, 48 hours) obtained from 5 healthy subjects. For sirolimus, a two compartment model was calculated using sirolimus dose and blood concentration values (blood concentration at t = 0, 2, 24, 48, 96 hours) obtained from the same 5 healthy subjects. Maximum likelihood estimates were sought for apparent clearance (CL/F) and apparent volume of distribution (Vd/F).

Study Arms and Samples

The drug treatment phase lasted three days for tacrolimus and sirolimus with the last dose taken on the morning of the fourth day. Peripheral whole blood samples were collected at the baseline visit prior to starting medications. For tacrolimus, concentrations were measured at timed intervals of 2 hrs and 48 hrs after final dose. Sirolimus concentrations were measured at 24, 48 and 96 hours after the final dose due to the long half-life of sirolimus. Tacrolimus and sirolimus levels were measured from whole blood using a microparticle enzyme immunoassay.

Mitogen Stimulation

Peripheral blood mononuclear cells were analyzed using a mitogen stimulation assay (Cylex™). This assay quantitates the level of intracellular ATP. A mitogen stimulant [phytohemagglutinin (PHA)] was added to whole blood samples and the cells were incubated at 37°C for 16–18 hours. Control samples were incubated without PHA. The CD4 T cells were purified with magnetic beads coated with anti-CD4 monoclonal antibody. The purified CD4 T cells were lysed to release intracellular ATP, which was read using a luminometer. [http://www.cylex.net/pdf/ImmuKnow_Insert-cx.pdf]. The assay results were normalized for area-under-the-curve for drug exposure. The results in the two arms were analyzed using paired t test to assess significant difference set at p value <0.05.

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Levels of IL-17A

IL-17A levels were assessed using peripheral blood CD4 T cells stimulated with anti-CD3/CD28. A commercially available ELISA kit was used to quantify the cytokines (per protocol provided by manufacturer eBioscience). A paired t test analysis was used to assess for significance.

Lymphocyte Proliferation

Lymphocyte proliferation was determined by incorporating BrdU [5-bromo-2-deoxyuridine] during DNA synthesis. The peripheral blood samples were processed with Ficoll-Paque to obtain mononuclear cells. The CD4 cells were isolated with an indirect magnetic labeling system and activated using Dynabeads coated with human anti-CD3/CD28 antibodies. The cells were cultured in a 96 well plate at 37°C for 48 hours. BrdU was added to the cultures for the last 6 hours of incubation. The absorbance was measured at 370 nm using an ELISA plate reader.

Urine Protein/Creatinine ratios

Urine samples were collected prior to the start of study, during administration of the drug, and one week after the completion of drug administration. These samples were evaluated for urine protein/creatinine ratios.

Statistical methods

Paired t test analysis was used to evaluate significant differences between groups. Analysis of variance was performed on the oral glucose tolerance test to determine if there was a significant change in glucose handling during the drug administration. Data was stored and analyzed in Microsoft Excel.

Results and Discussion

Results

Normal subjects were enrolled and all participated in two study arms: sirolimus and tacrolimus administration (Table 1). Drug effects on lymphocyte activation were determined by mitogen responses, proliferation, and cytokine production. These subjects had no underlying medical conditions and were not on any prescribed medications. There were no significant differences in blood pressure, baseline creatinine, or HgbA1c. The median age of subjects was 49. The systolic blood pressures of study subjects ranged from 101–124 mmHg. Average HgbA1c was approximately 5.4%. There were no dropouts and no observed complications during the study or at one month safety follow-up visit.

Pharmacokinetic parameters for sirolimus and tacrolimus were analyzed (Tables 2b). For the sirolimus group, the mean area under the curve (AUC) $AUC_{(0–24)}$ (ng*hr/mL) was 0.462 with a standard deviation of 0.125. The mean half-life for sirolimus was 63.7 hours with a standard deviation of 7 hours. For the tacrolimus group, the mean AUC was 0.220 with a standard deviation of 0.07. The mean half-life for tacrolimus was 40 hours with a standard deviation of 9 hours. The drug concentrations for tacrolimus were within the recommended

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clinical therapeutic range for all subjects (Fig 1a). The drug concentrations for sirolimus were also within clinically relevant therapeutic ranges (Fig 1b).

The effects of single-drug immunosuppression on lymphocyte proliferation were determined by BrdU incorporation (Fig 2). The BrdU assay at baseline, prior to administration of drug, was not significantly different for both arms of the study (Fig 2a). At 48 hours after the last dose of the drug was administered, lymphocyte proliferation was reassessed. The BrdU was normalized for drug exposure using AUC of drug level. The sirolimus group continued to exhibit decreased immune responses (Fig 2b). In contrast, the tacrolimus group exhibited rebound immune reactivity which was significantly higher than with sirolimus. Analysis of T cell activation, based on mitogen responses, suggests similar trend as with the BrdU analysis (Fig 2C).

T cell function was also assessed by measuring cytokine production (Fig 3). Peripheral blood T cells were stimulated with anti-CD3/CD28, and the cytokine IL-17A was analyzed. A significant decrease in IL-17A production in the group receiving sirolimus was detected. The IL-17A levels in the sirolimus arm were decreased at 24 hours but not at 48 hours. The tacrolimus group did not reveal a decrease in IL-17A either at 2 or 24 hours after the last dose.

Urine total protein/creatinine ratios were determined at baseline, prior to drug administration, during the trial, and at one week after completion of the trial. No significant change in the protein/creatinine ratios at these time points was noted (not shown). Drug effects on glucose tolerance were also analyzed. The oral glucose tolerance tests were compared using analysis of variance, and no significant difference was detected between tacrolimus and sirolimus groups (not shown).

Discussion

Current immunosuppressive regimens for transplant recipients involve multiple therapeutic agents, including a combination of steroids, calcineurin inhibitors, mTor inhibitors, mycophenolic acid, and occasionally azathioprine(11–13). Multidrug regimens have proven to be most effective in preventing graft rejection(11–13). Thus, most clinical studies analyze the effect of a cocktail of immunosuppressive agents.

To determine the immunosuppressive effects of a single agent (tacrolimus or sirolimus) we investigated the immune efficacy of each drug in a crossover study in normal subjects. Limitations of this study include a small number of participants and short duration of drug response. Nevertheless, using a crossover study design we detected a significant difference in lymphocyte proliferation and cytokine secretion in an effort to analyze single-drug effects.

Sirolimus (an mTOR inhibitor) and tacrolimus (a calcineurin inhibitor (CNI)) are two successful cornerstone drugs used to prevent graft rejection. The side effects of these drugs include high blood pressure, rash, decreased blood platelets, or, in the case of tacrolimus, nephrotoxicity or viral infections (cytomegalovirus), and hypertension. CNIs are used in the first month following transplantation with a reduction of CNI use for short and long term treatment(1, 2, 14, 15). Nephrotoxicity is a major problem accompanying CNI use that leads

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to acute and chronic renal failure. Sirolimus has been used as a rescue drug following unsuccessful CNI use.

For both sirolimus and CNIs, an additional consideration may be non-compliance of medication (16). Interestingly, Greenstein and Siegar examined questionnaires from 1547 renal transplant recipients, and found that patients' reasons for non-compliance included the belief that the transplant procedure had improved their health, not the medication(17).

We found a significant difference in lymphocyte proliferation between sirolimus and tacrolimus treatment. Results from the BrdU and Cylex™ assays indicate that sirolimus, in the absence of other immunosuppressants, exhibits reduced lymphocyte responsiveness with a trend toward continued suppression at 48 hours. In contrast, a similar effect is not observed with tacrolimus. This difference may be related to the longer half-life of sirolimus. No differences in proteinuria and oral glucose tolerance tests were detected.

We also observed that the half-life of tacrolimus is longer in healthy volunteers than reported in renal transplant recipients. The half-life for sirolimus is similar in both groups. The prolonged half-life of tacrolimus compared to previous reports may be due to concomitant medications or co-morbidities in transplant populations. In this study, the majority of the samples were obtained at the end of the dosing interval so the absorption rate constant (Ka) is fixed for some individual patients using literature values. The influence of co-variants such as weight, age, gender, and hematocrit were not assessed due to the limited sample size.

Prior studies regarding the impact of tacrolimus or sirolimus are variable. In *in vitro* studies, tacrolimus and anti-CD3/anti-CD28 activated T cells increased IL-2 mRNA (8 of 11 healthy subjects)(19). Kidney transplant patients (n=8) who had received tacrolimus or cyclosporine prior to transplant exhibited unaffected IL-2 mRNA concentrations (4 of 8 samples). Whole blood samples incubated with anti-CD3/anti-CD28 monoclonal antibodies with tacrolimus exhibited an increase in IL-2 mRNA(20).

Calcineurin inhibitors are a mainstay in immunosuppressive protocol in transplantation. In this study we did not observe inhibition of CD4 T cell proliferation and activation based on mitogen response or cytokine production. These results may be due to the short duration of drug exposure or to the limited effects of single-drug treatment. Our data suggest that tacrolimus may require longer treatment periods to induce immunosuppression. Long-term treatment periods may necessitate additional therapeutic modalities, such as induction with antibodies or high dose steroids during the post-transplant period. In contrast, sirolimus rapidly induced immunosuppressive effects. Future studies investigating the comparative effectiveness of sirolimus in a diverse patient group (e.g., adherent, partially-adherent) are warranted.

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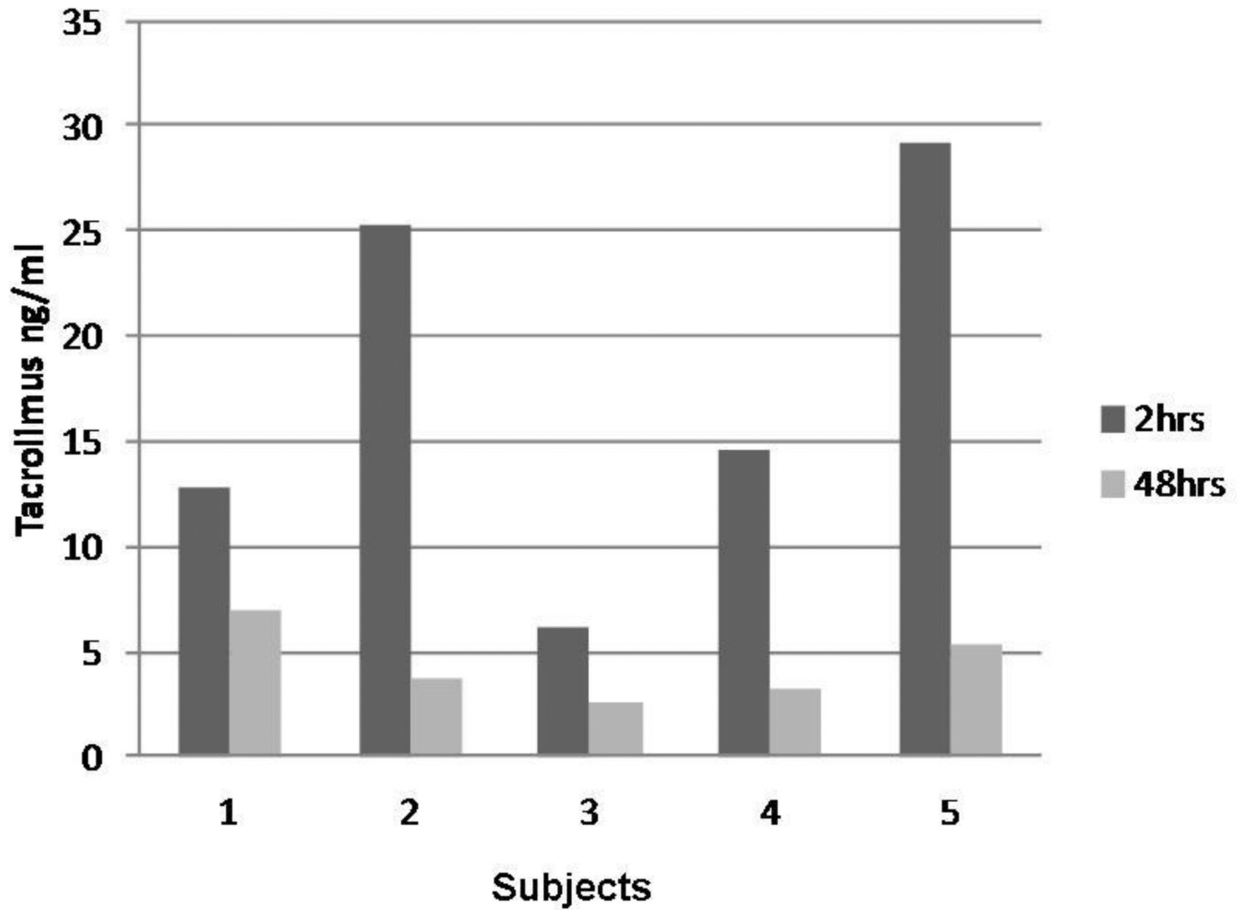

Figure 1a:
Tacrolimus concentrations: Participants received three and a half days of tacrolimus administration and doses were based on pharmacokinetic modeling for each individual subject. Serum samples were obtained from participants on day four, two hours following the last morning dose of tacrolimus (2 hrs.). A second serum sample was obtained 48 hours after last dose of tacrolimus (48 hrs.). Whole blood samples were analyzed using a microparticle enzyme immunoassay.

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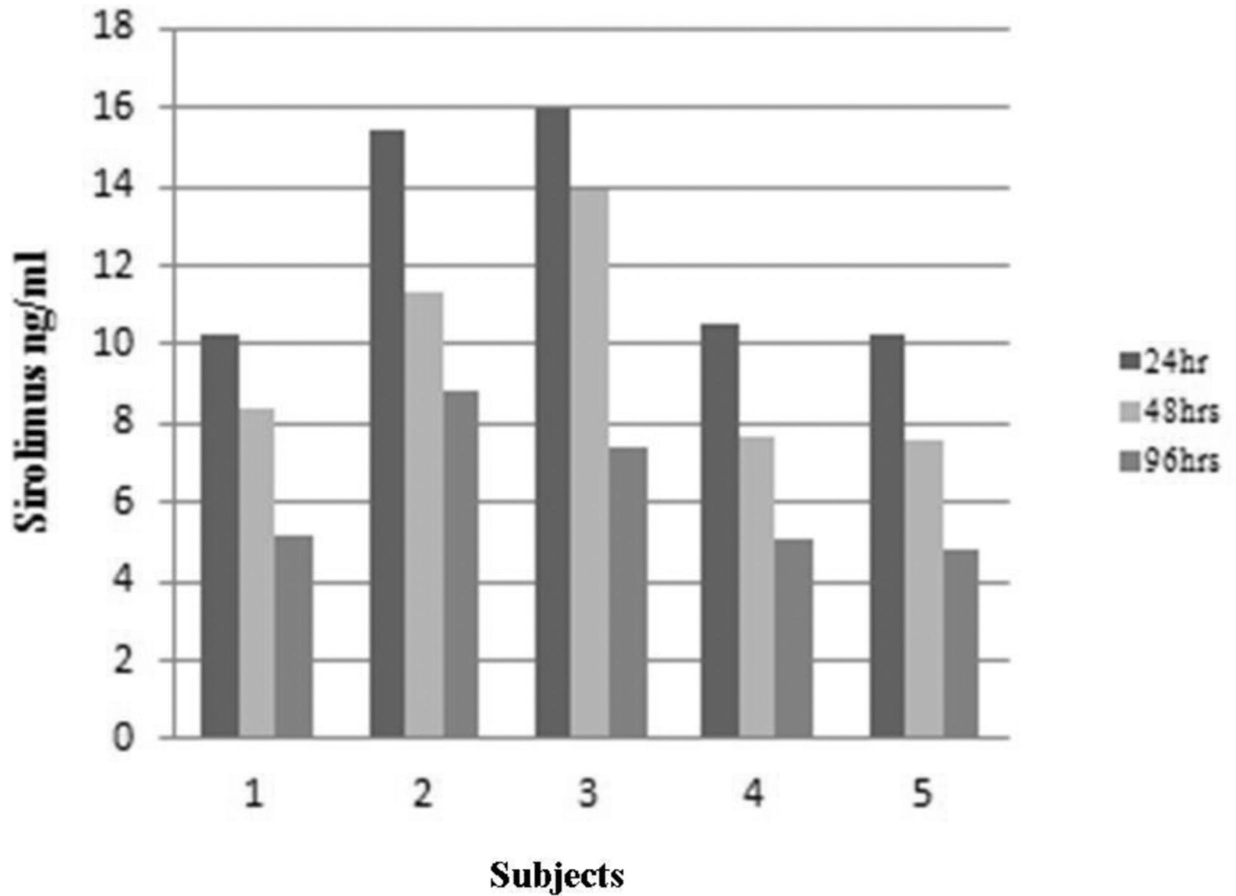

Figure 1b:
Sirolimus serum concentrations: Participants received four days of sirolimus administration and doses were based on pharmacokinetic modeling for each individual subject. Serum samples were obtained from participants on day four, two hours following the last morning dose of sirolimus (2 hrs.). Other samples were obtained 48 hours and 96 hours after last dose of sirolimus. Whole blood samples were analyzed using a microparticle enzyme immunoassay.

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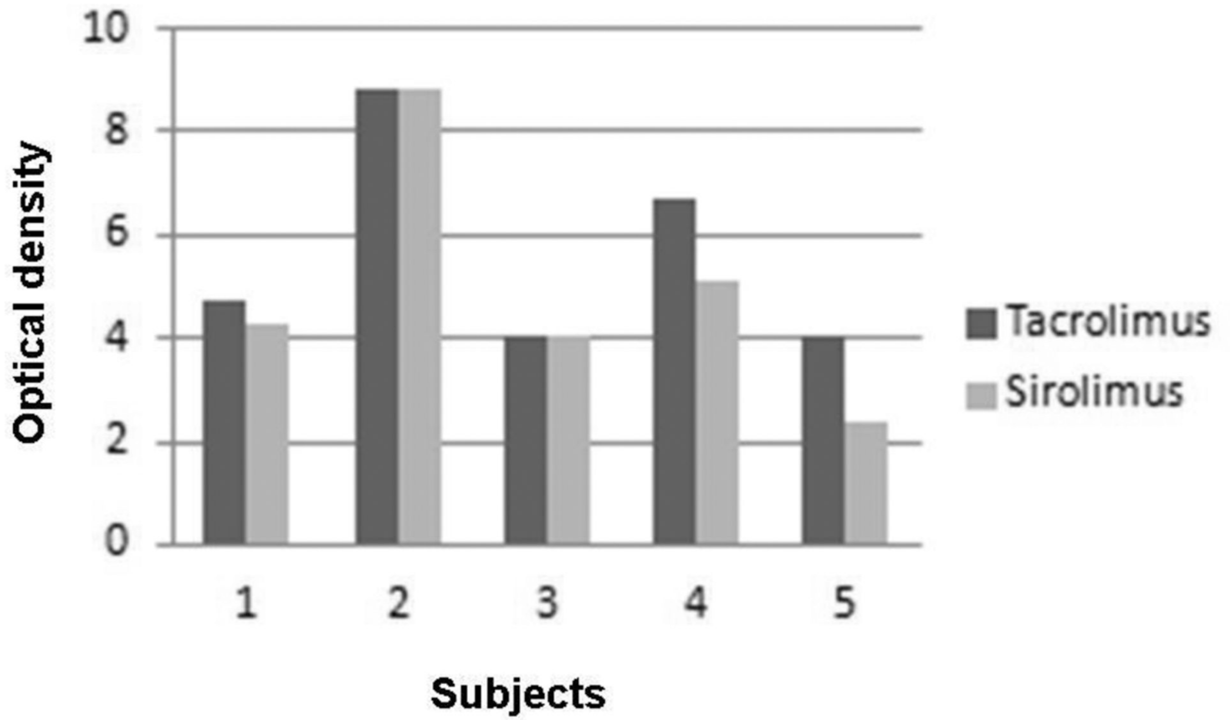

Figure 2a:

BrdU levels at baseline: Samples were processed for lymphocyte proliferation using incorporation of BrdU [5-bromo-2-deoxyuridine] during DNA synthesis. Blood samples were obtained from participants prior to the first dose of drug for both study arms (tacrolimus or sirolimus).

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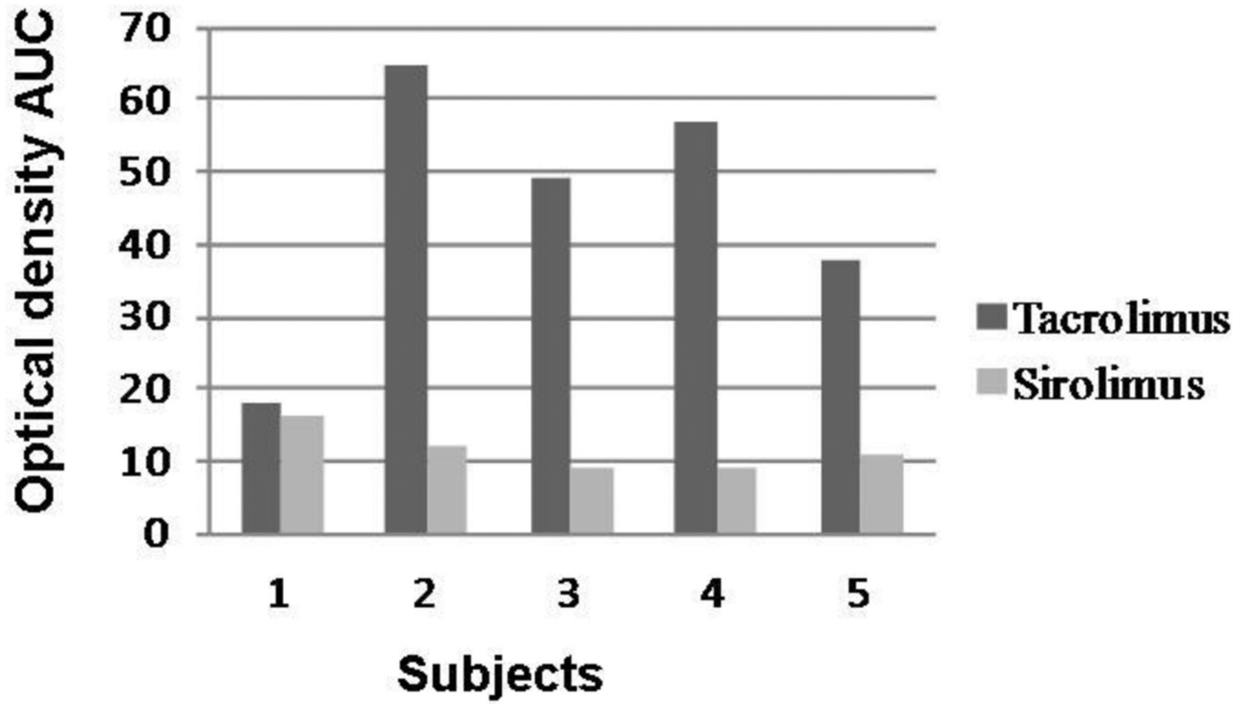

Figure 2b:
Lymphocyte proliferation. Samples were obtained at 48 hours after last dose of drug administration (three and a half days of bid dosing in the tacrolimus arm with last dose on day four, four days of daily dosing for sirolimus arm with last dose on day four). Lymphocyte proliferation was analyzed using incorporation of BrdU [5-bromo-2-deoxyuridine] during DNA synthesis. The results are normalized for area under the curve for drug exposure.

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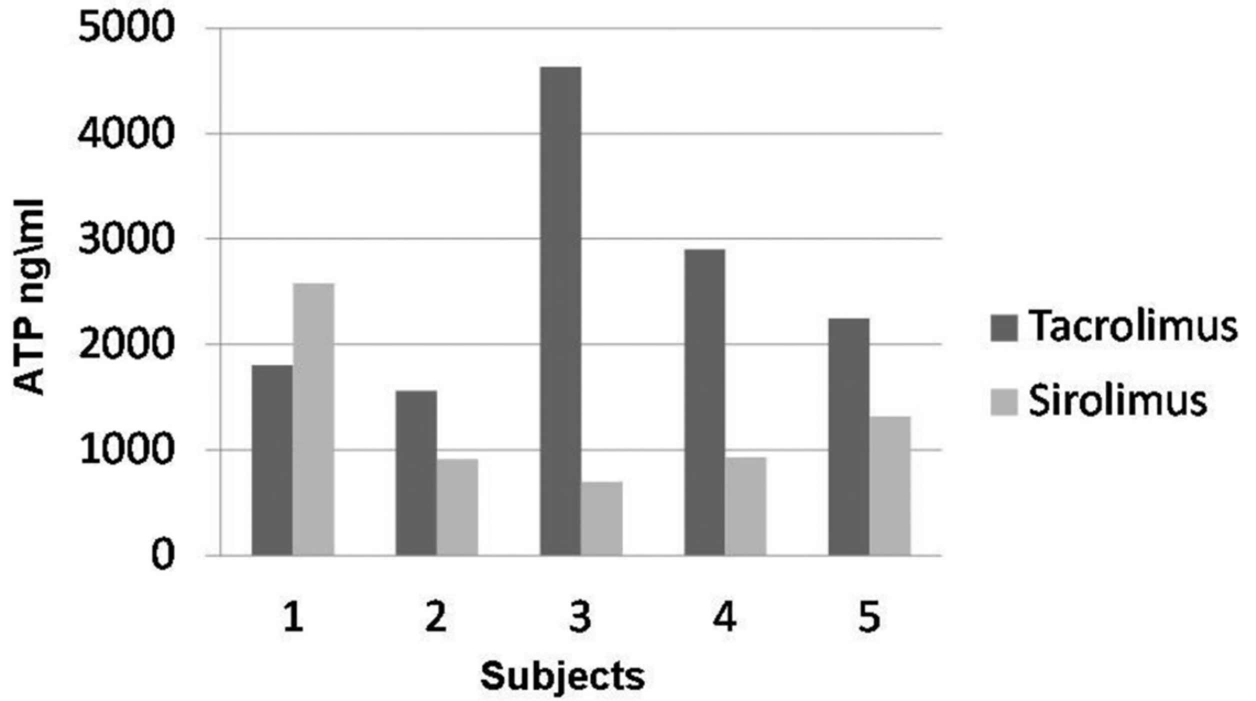

Figure 2c:

Cylex™ levels at 48 hours: Peripheral blood lymphocytes were analyzed for phytohemagglutinin (PHA) mitogen stimulation by the Cylex™ assay. Samples were obtained at the same time as samples for lymphocyte proliferation. These samples were obtained prior to start of drug administration and 48 hours after last dose. Additional samples were obtained for the sirolimus arm at 96 hours after the last dose.

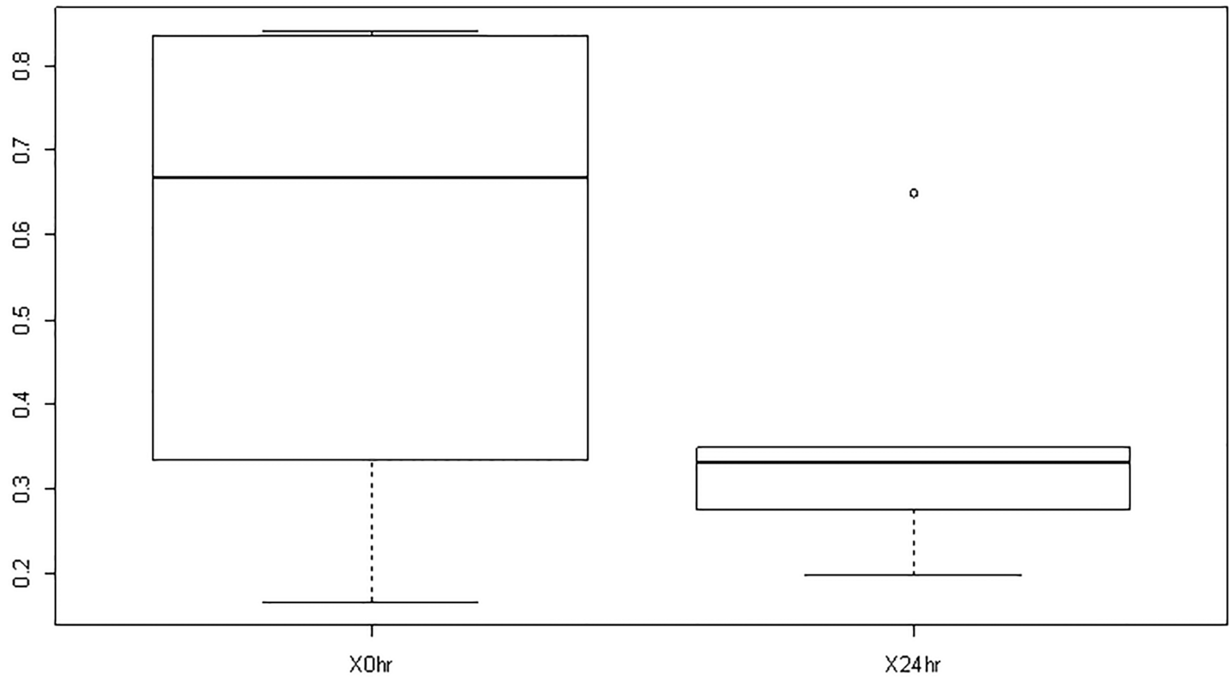

Figure 3a:
In arm one of the study, subjects were given sirolimus orally and serum IL-17A (a proinflammatory cytokine) levels were examined at 0 and 24 hours after administration ($p < .04$).

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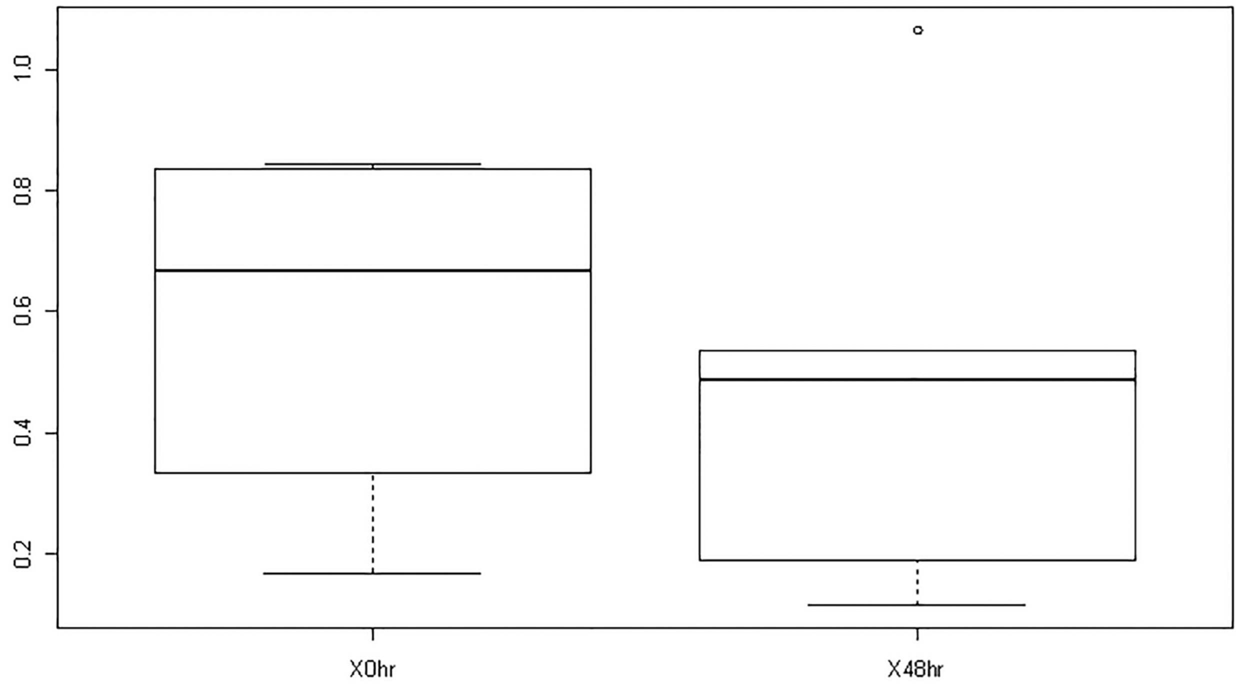

Figure 3b:

In arm one of the study, subjects were given sirolimus orally and serum levels of IL-17A were measured at 0 and 48 hours after last administration.

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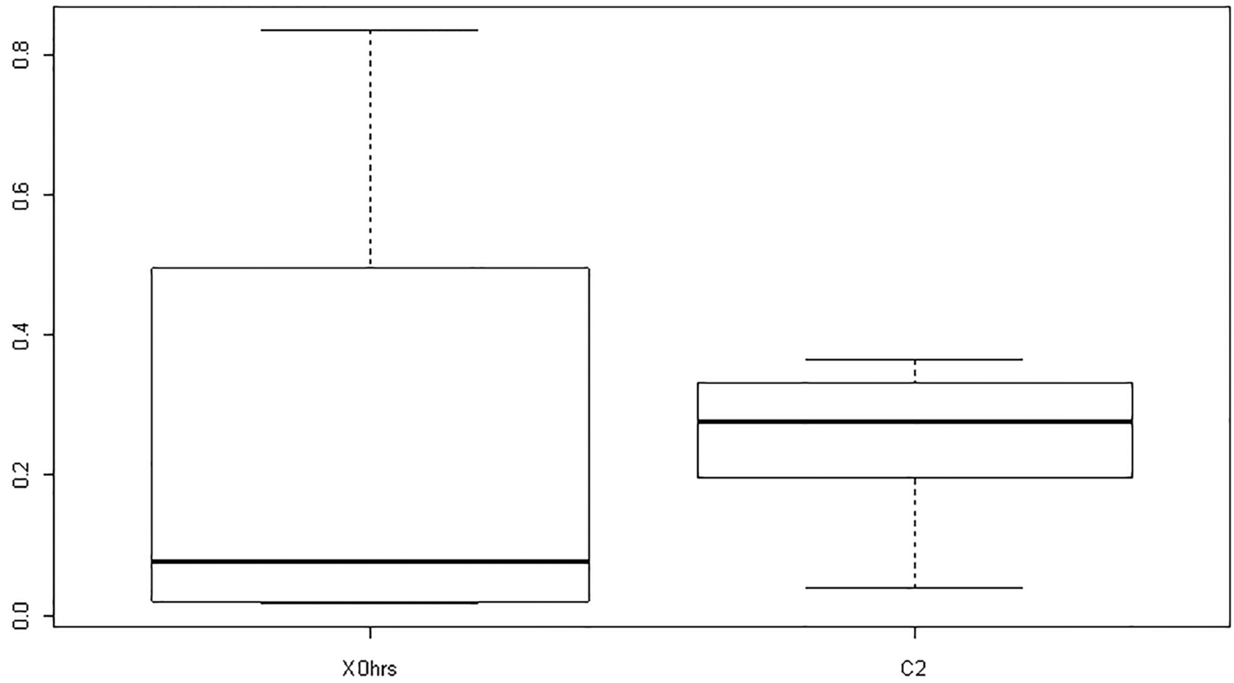

Figure 3c:
In arm two of the study, subjects were given tacrolimus orally and serum levels of IL-17A were evaluated at 0 and 2 hours following the last administration.

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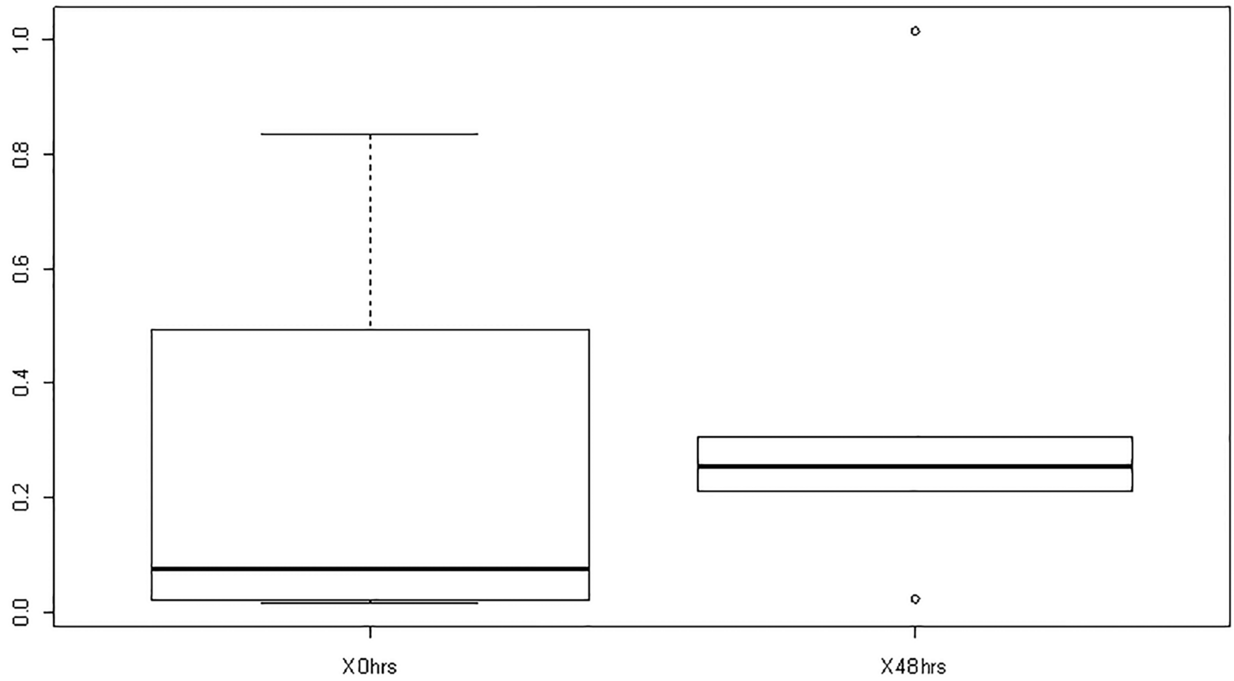

Figure 3d:
In arm two of the study, patients were given tacrolimus orally and serum levels of IL17A were measured at baseline (0 hours) and 48 hours after the last drug dose.

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Table 1:

Characteristics of subjects: No significant difference was detected in baseline blood pressure (BP), HgbA1c and creatinine and total cholesterol (Tchol). The baseline urine total protein/creatinine ratios ranged from 0.03–0.06 mg/dl. None of the participants had preexisting medical conditions or were taking prescription medications. BMI = body mass index, BP = blood pressure, Ur pr/cr = urine total protein/creatinine spot ratio, T Chol = total cholesterol

Subject	Age	Sex	Race	BMI	BP	Creatinine	Ur pr/cr	Hgb A1C	T Chol
1	33	M	Hispanic	29.2	113/66	0.90	0.04	5.6	185
2	49	M	Caucasian	28.6	120/78	0.88	0.04	5.5	226
3	50	M	Caucasian	29.4	124/81	1.02	0.03	5.6	240
4	54	M	Caucasian	24.8	105/72	0.72	0.06	5.5	188
5	19	F	Hispanic	26.3	101/60	0.71	0.04	5.2	191

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Table 2a:

Pharmacokinetic parameters of sirolimus concentration: Blood samples were obtained for analysis of sirolimus concentration. A two compartment model was calculated using the sirolimus dose and blood concentration values (blood concentration at t = 0, 2, 24, 48, and 96 hours). Maximum likelihood estimates were sought for apparent clearance (CL/F) and apparent volume of distribution (Vd/F). V2 = central compartment, V3 = peripheral compartment, k = elimination rate constant, k23 = distribution rate constant (central to peripheral), k32 = distribution rate constant (peripheral to central), CL = clearance, t1/2 = half-life, AUC = area under the curve

ID	DOSE (mg)	V2(L)	V3(L)	KA (hr-1)	K(hr-1)	K23(hr-1)	K32(hr-1)	CL (L/hr)	Tl/2 (hr)	$AUC_{(0-24)}$ (ng*hr/mL)
1	5.0	215.68	1008.60	0.38	0.08	0.27	0.06	17.20	59.60	0.29
2	6.0	100.11	837.71	0.32	0.11	1.25	0.15	10.85	64.10	0.55
3	5.0	42.60	618.26	0.18	0.19	0.75	0.05	8.18	68.70	0.61
4	3.0	88.92	472.82	0.32	0.08	0.20	0.04	7.05	71.90	0.43
5	4.0	71.98	582.65	0.49	0.13	0.88	0.11	9.35	54.30	0.43
Mean	4.6	103.86	704.01	0.34	0.12	0.67	0.08	10.52	63.70	0.46
Std	1.1	66.16	215.74	0.12	0.05	0.44	0.05	3.99	7.00	0.13

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Table 2b:

Pharmacokinetic parameters of tacrolimus concentration: Blood samples were obtained for analysis of tacrolimus concentration. A two-compartment model with first-order absorption pharmacokinetics was calculated using the tacrolimus dose and the blood concentration values (blood concentration at t = 0, 2, 12, 24, and 48 hours). V2 = central compartment, V3 = peripheral compartment, k = elimination rate constant, k23 = distribution rate constant (central to peripheral), k32 = distribution rate constant (peripheral to central), CL = clearance, t1/2 = half-life, AUC = area under the curve

ID	DOSE (mg)	V2(L)	V3(L)	KA (hr-1)	K(hr-1)	K23(hr-1)	K32(hr-1)	CL (L/hr)	t1/2 (hr)	$AUC_{(0-12)}$ (ng*hr/mL)
1	4.00	9.44	500.10	0.30	1.30	2.10	0.04	12.30	46.00	0.33
2	10.00	126.80	1194.00	0.11	0.35	0.23	0.03	43.76	48.00	0.23
3	2.00	19.60	602.64	0.21	0.70	1.38	0.05	13.73	46.50	0.15
4	3.00	65.59	547.12	0.66	0.29	0.82	0.10	18.92	28.90	0.16
	3.50	14.48	468.76	0.58	0.99	3.08	0.10	14.39	30.40	0.24
Mean	4.50	47.18	662.52	0.37	0.73	1.52	0.06	20.62	40.00	0.22
Std	3.20	49.83	301.37	0.24	0.43	1.11	0.03	13.17	9.40	0.07