Pharmacokinetic Interactions Augment Toxicities of Sirolimus/Cyclosporine Combinations

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Abstract. This study correlated the dynamic effects of sirolimus (rapamycin; RAPA) and cyclosporine (CsA) alone versus in combination to produce renal dysfunction, myelosuppression, or hyperlipidemia, with their corresponding blood and tissue concentrations. After salt-depleted rats were treated with RAPA (0.4 to 6.4 mg/kg per d) and/or CsA (2.5 to 20.0 mg/kg per d) for 14 d, the GFR, lipid levels, bone marrow cellularity, and CsA/RAPA concentrations in whole blood versus liver or renal tissues were measured, and the median effect model was used to discern the type of drug interactions. Compared with vehicle controls (1.98 ± 0.34 ml/min), GFR values were reduced only by large doses of drug monotherapy, namely RAPA (3.2 mg/kg per d = 1.2 ± 0.02 ml/min or 6.4 mg/kg per d = 1.3 ± 0.2 ml/min; both P < 0.01) or CsA (10.0 mg/kg per d = 1.2 ± 0.1 ml/min or 20.0 mg/kg per d = 0.8 ± 0.4 ml/min; both P < 0.01). In contrast, hosts that were treated with smaller doses of CsA/RAPA combinations showed more pronounced effects in reduction of GFR values: 2.5/0.4 mg/kg per d, modestly (1.5 ± 0.5 ml/min; P < 0.01); 5.0/0.8 mg/kg per d, moderately (0.23 ± 0.01 ml/min; P < 0.001); and higher-dose groups, markedly. The exacerbation of renal dysfunction seemed to be due to a pharmacokinetic interaction of RAPA to greatly increase CsA concentrations in whole blood and, particularly, in kidney tissue. In contrast, the pharmacodynamic effects of CsA to potentiate two RAPA-mediated toxicities—myelosuppression and increased serum cholesterol/low-density lipoprotein cholesterol—occurred independently of pharmacokinetic interactions. RAPA aggravates CsA-induced renal dysfunction owing to a pharmacokinetic interaction, whereas CsA produces a pharmacodynamic effect that augments RAPA-induced myelosuppression and hyperlipidemia.

The development of agents that produce synergistic immunosuppression with the calcineurin antagonists (CNA)—cyclosporine (CsA) or tacrolimus—is based on the hypothesis that drug-dose sparing mitigates renal dysfunction, as well as the pleiotropic neural and hepatic toxicities associated with CNA administration (1,2). Furthermore, the addition of a synergistic drug may reduce the adverse impact of the large variations in the pharmacokinetic (3,4) and pharmacodynamic (5) behavior of CsA among renal transplant recipients. Although the addition of sirolimus (rapamycin; RAPA) to a CsA-based regimen has fulfilled the expectations of synergistic immunosuppression (6,7), randomized, blinded trials document inferior renal function, compared with CsA/azathioprine (Aza)/prednisone (Pred)- or CsA/placebo/Pred-treated patients (8,9). However, CsA and RAPA show a pharmacokinetic interaction that is due, at least in part, to common metabolism by cytochrome P450 3A4. Therefore, the present study used an animal model to dissect the pharmacokinetic from the pharmacodynamic components of the toxicity produced by CsA/RAPA combinations.

Although the exact mechanisms of CsA-induced nephrotoxicity are not understood fully, important components include increased vascular resistance, which produces decreased renal blood flow (10,11); generation of reactive free radicals, which causes both oxidative stress (12) and cytochrome P450 activation (13); upregulated expression of the profibrogenic principle transforming growth factor-β (14); increased generation and responses of smooth muscle cell calcium to vasoconstrictive stimuli (15); upregulated synthesis and expression of angiotensin II receptors (15); and depressed nitric oxide production by both endothelial and inducible nitric oxide synthases (16). Furthermore, CsA has been reported to promote Fas-mediated (17) apoptosis of LLC-PK1-cultured renal tubular cells in vitro, an effect that is blocked by peptide inhibitors of caspases 3, 8, and 9 (18). Thus, increased vasoconstriction and apoptosis characterize CsA nephrotoxicity.

Because the synergistic immunosuppressive interactions between CsA and RAPA were first documented in rats (19,20), this species represented a logical model to investigate the...
impact of the drug combination on the kidney. The salt-depleted rat model has been reported to produce functional and histopathologic effects that resemble the dose-dependent impairment of renal function observed in CsA-treated kidney transplant patients (1,10,21–23). In contrast, administration of therapeutic doses of RAPA (0.04 to 0.8 mg/kg per d intravenously) caused no significant renal changes in rats (24). At 0.8 mg/kg per d RAPA, normal rats displayed only a marginal elevation in serum creatinine (SCr) values, and spontaneously hypertensive rats showed neither accelerated necrotizing vasculopathy nor tubular atrophy (25). Only supratherapeutic (1.6 to 6.4 mg/kg per d) RAPA doses produced transmural fibrinoid necrosis of vessels in the gastrointestinal submucosa and in the kidney, as well as juxtamedullary hypertrophy, tubular dilation, basement membrane thickening, vacuolization, and atrophy (25).

Owing to its use in combination with CNA, there is considerable interest in understanding the impact of RAPA on CsA-induced nephrotoxic injuries. Andoh et al. (22) reported that subcutaneous coadministration of RAPA potentiated CsA-induced nephrotoxicity in salt-depleted rats and postulated that the effect was due to enhanced hyperglycemia. In contrast, we suggested that increased drug concentrations contribute to the adverse renal effects displayed by the CsA/RAPA combination, because we had documented elsewhere that a portion of the synergistic effect was due to pharmacokinetic interactions (26), particularly after oral coadministration (27). The present study revealed a predominant role of pharmacokinetic interactions to produce toxic renal exposures of CsA and of dynamic effects to potentiate the lipid as well as the myelosuppressive toxicities.

Materials and Methods

Animals

Male Wistar Furth (RT1<sup>+</sup>) rats (160 to 200 g), obtained from Harlan Sprague Dawley (Indianapolis, IN), were housed in cages in a temperature- and light-controlled environment. The animals, which were maintained ad libitum on either regular or low-salt chow (0.05% sodium; Teklad Premier, Madison, WI) with access to tap water, were weighed and examined daily.

Drugs

Commercial oral formulations of CsA (Sandimmune; Novartis Research, East Hanover, NJ) and RAPA (Rapamune; Wyeth-Ayerst, Princeton, NJ) were administered by oral gavage in a constant volume of 0.2 ml daily for 14 d.

Experimental Groups

After a 7-d conditioning period on low-salt chow, groups of six rats were assigned randomly to treatment for 14 d with CsA alone (2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 mg/kg per d), RAPA alone (0.4, 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d), or CsA/RAPA combinations at a fixed 6.25:1 ratio (2.5/0.4, 5.0/0.8, 7.5/1.2, 10.0/1.6, 15.0/3.2, or 20.0/6.4 mg/kg per d), which had been shown to be the optimal ratio to document synergistic immunosuppression, or at varying ratios of fixed 5.0 or 10.0 mg/kg per d CsA doses with ascending amounts of RAPA (0.4, 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d). In addition, there were two untreated control groups, each composed of six rats: one fed a low-salt diet and the other fed a normal diet.

After receiving the final drug doses on day 14, the animals were placed in metabolic cages for 24-h urine collections and GFR measurements, by use of iohexol in the Renalyzer PRX 90 (Provalid AB, Lund, Sweden). Inman et al. (28) documented in rats that this method provides an accurate and reliable measure of GFR, compared with inulin determinations. In addition, urinary sodium, potassium, magnesium, calcium, phosphate, and creatinine levels were quantified by use of established methods in our clinical chemistry laboratory. Upon completion of the urine collection, the animals were anesthetized with intraperitoneal pentobarbital (Abbott, Chicago, IL) to obtain whole-blood samples for complete blood counts, CsA and RAPA concentration measurements, and serum aliquots for sodium, potassium, magnesium, calcium, phosphate, uric acid, cholesterol, creatinine, and lipoproteins. The laboratory results, presented as mean values ± deviants, were compared for statistical significance by use of ANOVA; P < 0.05 was accepted as significant.

The left kidney was removed and split in half. One half of the left kidney and a 2-g sample of the right lateral lobe of the liver were used for drug concentration measurements. The other half of the left kidney was fixed in buffered 10% formalin and processed overnight. 3-µm histologic sections were stained with progressive hematoyxin-eosin, periodic acid-Schiff, or Masson’s Trichrome reagents. Two independent pathologists (J.C. and R.V.), who were blinded to treatment assignments, used semiquantitative scales of light microscopic criteria to assess the degree of vasculopathy, glomerular changes, and tubulointerstitial damage in multiple kidney sections. Tubular and glomerular changes were graded separately as follows: 0, no changes; 1+, <5%; 2+, 5 to 25%; 3+, 25 to 50%; and 4+, >50% involvement. A similar vascular scale included the following: 0, none; 1+, minimal; 2+, mild; 3+, moderate; and 4+, severe. Although the scores generally were concordant, when they were disparate, a mean value was chosen as the histopathologic grade.

Renal Function

GFR was measured by the iohexol method (29). The femoral vein and artery, as well as the transplant ureter, were cannulated individually by use of 10-0 silicone tubing (Baxter, Deerfield, IL). BP, heart rate, and urine output were monitored with the use of a Micro-Med apparatus (Louisville, KY) and analyzed with the use of a DMSI 2004 computer program (Micro-Med). BP was recorded automatically every 30 s and urine output every 5 min. A loading dose of 1000 mg/kg iohexol (Omnipaque, 300 mg/ml; Nycomed, Inc., Princeton, NJ) was administered intravenously over 5 min, followed by infusion of 600 mg/kg over 90 min, as recommended by Inman et al. (28). Urine samples, collected at 20-min intervals after completion of the loading dose, were analyzed for iohexol concentrations. Whole-blood samples were obtained at the midpoints of the urine collections. GFR values (ml/minute) were calculated by the formula (U × V)/P, where U is urinary iohexol concentration (mg/ml), V is urine output (ml/24 h), and P is plasma iohexol concentration (mg/ml). The results were presented as mean ± deviants, and statistical significance was assessed by t test.

Bone Marrow Cellularity

The right femur was harvested, fixed in 10% buffered formalin, decalcified in formic acid for approximately 1 wk, sectioned (3 to 5 µm), and stained with hematoxylin and eosin by use of standard techniques. Hematopoiesis was estimated as the percentage of the marrow space occupied by cellular as opposed to adipose tissue.
elements. The average number of megakaryocytes in four high-power fields (40×) was used to estimate the effects of RAPA with or without concomitant administration of CsA on platelet formation.

**Drug Concentration Measurements**

For CsA measurements, whole-blood samples (0.5 ml) were collected into ethylenediaminetetraacetate-containing tubes (Becton Dickinson, Mountain View, CA); 1- to 2-g aliquots of hepatic and renal tissues were disrupted by use of an Ultrasound Homogenizer (Fisher Scientific, Pittsburgh, PA). CsA determinations were performed by use of an automated fluorescence polarization immunoassay (TDx; Abbott, Chicago, IL). In contrast to human specimens and on the basis of supporting data of others (30–35), we documented elsewhere that the TDx technology provides results similar to HPLC because rats do not produce CsA metabolites that cross-react significantly in the TDx assay (36). CsA concentrations were expressed as ng/ml for whole blood or ng/g for wet-tissue weight. The intra-/interassay coefficients of variation for blood and tissue CsA measurements were 3.2% at 150 ng/ml and 1.7% at 800 ng/ml, and 2.3% at 150 ng/ml and 1.6% at 800 ng/ml, respectively (Napoli KL, Kahan BD, unpublished observations).

RAPA concentrations were estimated by use of our published method of HPLC with ultraviolet detection (37). Owing to the photosensitivity of RAPA, left kidney and liver samples (1 to 2 g) had to be protected from light during ultrasonic disruption. Briefly, 1 ml of 0.1 M sodium carbonate and 20 ml of methanolic-estradiol-3-methyl ether, an internal standard, were added to 1 ml of whole blood. After double extraction with 10 ml of t-buty methyl ether, the pooled supernates were evaporated, reconstituted twice with 150 μl of absolute ethanol, and finally suspended in 100 μl of mobile phase buffer composed of an 85:15 ratio of methanol/water. After centrifugation, 85-μl aliquots of supernates were injected onto tandem Supelcosil C18 columns (Supelco, Bellefonte, PA) heated to 40°C. During elution at a flow rate of 0.5 ml/min, ultraviolet absorbance was monitored at 276 nm. RAPA concentrations were estimated on the basis of a calibration curve consisting of 8 drug-free whole-blood (or tissue) samples that

![Figure 1](https://example.com/Figure1.png)

*Figure 1.* Effect of cyclosporine (CsA) and rapamycin (RAPA) alone or in combination on animal weight and renal function. Animals that were fed a low-salt diet either were untreated (■) or were treated for 14 d with CsA alone at doses of 2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 mg/kg per d (●); RAPA alone at doses of 0.4, 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d (□); or CsA/RAPA combination at doses of 2.5/0.4, 5.0/0.8, 7.5/1.2, 10.0/1.6, 15.0/3.2, or 20.0/6.4 mg/kg per d (▲). In addition, some rats were treated with a constant dose of 5 (□) or 10 (▲) mg/kg per d CsA with ascending RAPA doses, namely 0.4, 0.8, 1.2, 1.6, 3.2, and 6.4 mg/kg per d. After 14 d, we measured percentage of weight change in comparison with the weight at the beginning of therapy (A), 24-h urine output (B), serum creatinine levels (SCR; C), and GFR (D). For details, see Materials and Methods section.
had been spiked with 0, 2, 5, 10, 20, 30, 40, or 50 ng of RAPA. The assays for tissue concentrations added 0.5 ml of drug-containing (or, for calibrators, exogenously spiked) homogenates to 0.5 ml of sodium carbonate. The intra-/interassay coefficients of variation for blood and tissue RAPA measurements were 6.4% at 4.0 ng/ml and 4.2% at 32 ng/ml, and 7.8% at 4.0 ng/ml and 5.6% at 32 ng/ml (38).

Statistical Analyses

Because all immunosuppressive agents studied to date (39) have obeyed the median-effect equation of Chou and Talalay (40), which relates dose (or concentration) to biologic effect, this model was chosen to assess the nephrotoxic interactions between CsA and RAPA. The relationship is described by the following equation:

\[
\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m
\]

where \(fa\) is the fraction affected, the percentage of inhibition (reduction from the normal value), \(fu\) is the uninhibited fraction \((1 - fa)\), \(D\) is the administered drug dose (concentration), \(Dm\) is the dose (concentration) required for 50% inhibition (the median effect), and \(m\) is

Figure 2. Pharmacokinetic interactions between CsA and RAPA. Whole-blood levels of CsA (A) and RAPA (B), kidney tissue levels of CsA (C) and RAPA (D), and liver tissue levels of CsA (E) and RAPA (F) were measured after a 14-d course of immunosuppressive therapy with CsA and RAPA alone or in combination. Experimental groups are described in Figure 1 legend.
The interaction between RAPA and CsA was assessed by a combination index (CI) analysis, which assessed the dose of each drug necessary to achieve x% inhibition:

$$\text{CI} = \frac{D_{IC} + D_{DC}}{D_{IA} + D_{DA}} + \frac{D_{IC} \times D_{DC}}{D_{IA} \times D_{DA}}$$

(3)

where $D_{IC}$ and $D_{DC}$ are the doses (or concentrations) of drugs when used in combination and $D_{IA}$ and $D_{DA}$ are the corresponding doses (or concentrations) of drugs used alone. CI values <1 reflect synergistic interactions, CI values = 1 reflect additive interactions, and CI values >1 reflect antagonistic interactions.

**Results**

**Body Weight Changes**

Control (untreated) animals that were maintained on a low-salt diet for 21 d showed a mean weight gain of 41.5 ± 22.8% (Figure 1A). In contrast, hosts that were treated with 2.5 or 5.0 mg/kg per d CsA showed less weight gain, and those that were given 10.0, 15.0, or 20.0 mg/kg per d showed a maximal weight loss of 10% (versus untreated hosts; all groups, $P < 0.001$). Although animals that were treated with 0.4 mg/kg per d RAPA showed a slight weight gain, those that received 0.8, 1.2, 1.6, 3.2, or 6.4 mg/kg per d RAPA showed a maximal weight loss of 20% (all groups, $P < 0.001$). Rats that were treated with CsA/RAPA drug combinations displayed a maximal weight loss of 27% (all groups, $P < 0.005$). The weight loss seemed more likely to reflect metabolic causes than anorexia, because all experimental animals had food in their stomachs and stool in their colons. Furthermore, there was no evidence of drug-induced diarrhea or dehydration, as evident on examination and by blood chemistries (vide infra).

**Renal Function Changes**

Animals that were treated with either CsA or RAPA monotherapy showed greater urine output than hosts in the control groups (Figure 1B; $P < 0.002$), suggesting the presence of renal injury. Because blood glucose levels were similar among rats in each group, there was no evidence that hyperglycemia was producing a diuretic effect. In contrast to the control group, which showed a mean SCr value of 0.25 ± 0.05 mg/dl, treatment with ascending 2.5 to 20.0 mg/kg per d doses of CsA produced serial increases in SCr values (all groups, $P < 0.0007$; Figure 1C). In contrast, rats that were treated with the smaller RAPA doses (0.4, 0.8, or 1.6 mg/kg per d) showed insignificant changes; only animals that received 3.2 or 6.4 mg/kg per d showed significantly increased SCr values ($P = 0.001$). The CsA/RAPA groups showed higher SCr concentrations than either monotherapy group, increasing from 0.35 ± 0.05 mg/dl for the 2.5/0.4 mg/kg per d group to 2.35 ± 0.37 mg/dl for the 20.0/6.4 mg/kg per d group. Interestingly, ascending RAPA doses added to fixed amounts of CsA (5 or 10 mg/kg per d) produced less adverse effects ($P = 0.005$) than those observed in groups with simultaneously increasing doses of both drugs ($P = 0.001$).

The SCr results were confirmed by the GFR values. In comparison to the normal GFR values (1.98 ± 0.34 ml/min; Figure 1D), animals that were treated with CsA doses of 5.0 mg/kg per d (1.1 ± 0.2 ml/min), 7.5 mg/kg per d (0.92 ± 0.37
ml/min), or 20.0 mg/kg per d (0.75 ± 0.24 ml/min) displayed significantly reduced GFR values. Although RAPA alone caused only modest effects, the GFR values were significantly lower among CsA/RAPA combination groups. Thus, RAPA potentiates the dose-dependent nephrotoxicity of CsA (CsA versus CsA/RAPA, P < 0.025 and RAPA versus CsA/RAPA, P < 0.0045).

**Blood and Tissue CsA and RAPA Concentrations**

Twenty-four h after the last dose of a 14-d course of therapy, CsA and RAPA concentrations were measured in whole blood as well as in liver and kidney tissues. Whole-blood trough concentrations increased proportionate to the CsA dose. At each dose level, concomitant administration of RAPA produced a significant pharmacokinetic interaction to increase further the CsA concentrations by approximately twofold above those found in hosts that were treated with CsA alone (Figure 2A; all, P < 0.006).

Similarly, the whole-blood concentrations of RAPA, which displayed dose-dependent increases, were exaggerated when the drug was administered concomitantly with CsA (Figure 2B). However, kidney and liver tissue drug concentrations

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**Table 1. Median effect analysis of CsA/RAPA-induced nephrotoxicity**

<table>
<thead>
<tr>
<th>Drug Measurement</th>
<th>Serum Creatinine Levels</th>
<th>GFR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dm/Cm</td>
<td>r</td>
</tr>
<tr>
<td><strong>Dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>19.85 mg/kg</td>
<td>0.92</td>
</tr>
<tr>
<td>RAPA</td>
<td>109.18 mg/kg</td>
<td>0.76</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Blood concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>5921.0 ng/ml</td>
<td>0.91</td>
</tr>
<tr>
<td>RAPA</td>
<td>45.16 ng/ml</td>
<td>0.65</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Kidney concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>3757.0 ng/g</td>
<td>0.90</td>
</tr>
<tr>
<td>RAPA</td>
<td>85.50 ng/g</td>
<td>0.77</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> The median effect equation was used to assess drug interactions, as described in Materials and Methods section. Dm, the dose (mg/kg per d) that produces 50% of the maximal effect; Cm, (ng/ml) the concentration necessary to produce the effect; CsA, cyclosporine; RAPA, rapamycin.

<sup>b</sup> The interaction between the drugs was assessed by the combination index (CI) analysis of the doses necessary to achieve x% inhibition: CIx = D1 combined/Dx1 alone + D1 combined/Dx2 alone + (D1 combined) (D2 combined)/[Dx1 alone][Dx2 alone]; CI <1 reflect synergistic interaction; CI = 1 demonstrates additive interaction; and CI >1 shows antagonistic interaction. Results are presented as mean ± SD with minimal and maximal range, as calculated using Microsoft Excel. Interactions were qualified as strongly synergistic (SS) with CI <0.1; synergistic (S) with CI 0.11 to 0.7; antagonistic (AN) with CI >1.2; or additive (AD) with CI = 0.71 to 1.19.

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**Table 2. Median effect analysis of CsA/RAPA effect on blood parameters**

<table>
<thead>
<tr>
<th>Drug Measurement</th>
<th>Serum Sodium</th>
<th>Serum Phosphate</th>
<th>LDL&lt;sup&gt;a&lt;/sup&gt; Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dm/Cm</td>
<td>r</td>
<td>CI Range</td>
</tr>
<tr>
<td><strong>Dose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>49.4 mg/kg</td>
<td>0.93</td>
<td>—</td>
</tr>
<tr>
<td>RAPA</td>
<td>11.8 mg/kg</td>
<td>0.92</td>
<td>—</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>2.2–0.6 (AD)</td>
<td>1–0.003 (S)</td>
<td>0.95–0.007 (S)</td>
</tr>
<tr>
<td><strong>Blood concentration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>8560.0 ng/ml</td>
<td>0.88</td>
<td>—</td>
</tr>
<tr>
<td>RAPA</td>
<td>69.7 ng/ml</td>
<td>0.98</td>
<td>—</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>2.4–0.6 (AD)</td>
<td>1–0.03 (S)</td>
<td>1.8–0.05 (AD/S)</td>
</tr>
<tr>
<td><strong>Kidney concentration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>56.98 ng/g</td>
<td>0.86</td>
<td>—</td>
</tr>
<tr>
<td>RAPA</td>
<td>1332.0 ng/g</td>
<td>0.88</td>
<td>—</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>2–1 (AD)</td>
<td>1.5–0.04 (AD/S)</td>
<td>1.9–0.07 (AD/S)</td>
</tr>
</tbody>
</table>

<sup>a</sup> LDL, low-density lipoprotein.
A median effect analysis was performed to examine rigorously dose- and concentration-dependent effects of CsA and/or RAPA on renal function, expressed as SCr levels or GFR (Table 1). The Pearson’s correlation coefficient values documented a good relation between drug doses and the median effect model for both SCr and GFR values (CsA \( r = 0.92 \), RAPA \( r = 0.76 \) and CsA \( r = 0.90 \)/RAPA \( r = 0.97 \), respectively). The median effect analysis also showed good correlations between kidney tissue concentrations and SCr levels or GFR (CsA \( r = 0.90 \)/RAPA \( r = 0.77 \) or CsA \( r = 0.85 \)/RAPA \( r = 0.98 \), respectively). Although the CI values that were calculated on the basis of drug doses suggested a synergistic interaction between CsA and RAPA, those that were based on whole-blood concentrations, which were markedly increased by the drug combination, showed less synergism and those that were based on kidney tissue concentrations demonstrated pharmacodynamic antagonism. These findings suggest that the alteration in renal function associated with CsA/RAPA dual-drug therapy primarily represents a pharmacokinetic interaction.

**Renal Analytes**

Serum magnesium, uric acid, and phosphate concentrations were measured as potential indicators of altered tubular function. In a dose-dependent manner, CsA but not RAPA reduced serum magnesium concentrations (\( P < 0.00001 \); data not shown). Interestingly, virtually all animals in the CsA/RAPA combination groups displayed magnesium concentrations similar to those of hosts that were treated with RAPA alone. Uric acid concentrations were increased by CsA monotherapy, especially at 15 and 20 mg/kg per d doses, although not significantly by RAPA alone. However, all animals in the CsA/RAPA combination groups displayed a dose-dependent increase in serum uric acid concentrations even greater than that for animals that were given CsA alone (\( P = 0.02 \) to 0.002; data not shown). A similar picture was observed for phosphate levels: only hosts that were treated with high doses of CsA alone displayed increased phosphate levels, whereas animals that received combination therapy displayed a dose-dependent increase in this analyte. When corrected for renal tissue concentrations, the effect of the drug combinations was found to be predominantly additive; only serum phosphate concentrations showed synergistic interactions (Table 2). Combination therapy had no significant impact on serum sodium (Table 2) or serum potassium concentrations (Figure 5). Furthermore, the dose-dependent increase in blood glucose levels produced by CsA but not by RAPA monotherapy was only slightly greater among the combination treatment groups. Overall, pharmacokinetic interactions of RAPA to increase renal tissue CsA concentrations readily accounted for the observed changes in renal analytes.

**Renal Histology**

Kidneys from rats that were given a low-salt diet alone or treated with the smallest drug doses showed scant evidence of pathologic abnormalities (Figure 6). Ascending doses from 0.8 to 6.4 mg/kg per d RAPA alone or 5.0 to 20.0 mg/kg per d CsA alone were associated with progressive tubular and glomerular abnormalities, as well as arterial wall thickening. Renal sections from hosts that were treated with the 10 mg/kg per d CsA dose showed increased glomerularcellularity accompanied by modest (<25%) thickening of vessels, focal tubular dilation,
and significantly increased interstitial fibrosis. The next higher CsA dose (15 mg/kg per d) caused significant arteriolar thickening, as well as focal necrotic changes, tubular dilation, and mild inflammatory cell infiltrates. The highest CsA dose (20 mg/kg per d) was associated with arteriolar vacuolization and hyalinosis, glomerular hypercellularity, tubular dilation, and diffuse interstitial inflammation and fibrosis. In contrast, only moderate changes in renal histopathology were present even at the highest RAPA dose (6.4 mg/kg per d).

Although kidneys from rats that were treated with the combination of 2.5 mg/kg per d CsA and 0.4 mg/kg per d RAPA did not show tubular or glomerular changes, they did display focal vasculitis, including cellular infiltration into the walls of medium- to large-sized vessels. Increasing doses of the CsA/
RAPA combination (5.0/0.8, 7.5/1.2, 10/1.6, 15/3.2, and 20/6.4 mg/kg per d) were associated with progressive tubular damage and thickening of the walls of small arterioles, as well as perivascular infiltration—changes that were far more pronounced than those observed among hosts that were treated with either drug alone. Similar changes were observed among rats that were treated with escalating doses of RAPA (0.4 to 6.4 mg/kg per d) in combination with fixed 5.0 or 10.0 mg/kg per d CsA (not shown). Thus, the addition of RAPA to a CsA regimen accelerates the appearance of histopathologic findings, confirming and extending the observations on alterations of GFR and SCr values.

Effects of CsA and RAPA alone or in Combination on Bone Marrow Hematopoiesis

Bone marrow cellularity was estimated as the ratio of space occupied by cellular versus adipose elements (Figure 7A). In comparison with the 97.66 ± 2.25% value for cellularity in untreated rats on a low-salt diet, even the highest doses of CsA (10 to 20 mg/kg per d) displayed little change (95.33 ± 2.94% to 87.5 ± 4.18%). RAPA alone (0.8 to 6.4 mg/kg per d) caused only a slight dose-dependent decrease in cellularity (92.83 ± 2.63% to 85.0 ± 6.32%; all, P < 0.001). In contrast, hosts that were treated with CsA/RAPA combinations displayed more profound reductions in cellularity, namely to 67.16 ± 26.76% at 10.0/1.6 mg/kg per d and to 54.16 ± 30.72% at 20.0/6.4 mg/kg per d (CsA/RAPA versus CsA [P < 0.004] versus RAPA [P < 0.005]). A similar decrease in bone marrow cellularity was observed when fixed amounts of CsA (5 or 10 mg/kg per d) were combined with ascending RAPA doses (all, P < 0.001). These interactions were modestly synergistic, whether the analysis was performed on the basis of drug doses or of renal (or liver) concentrations as a surrogate for bone marrow levels (Table 3).

Control rats on a low-salt diet displayed 14.8 ± 3.3 megakaryocytes (MEG) per high-power (40×) field (HPF). Monotherapy with 10.0 mg/kg per d CsA significantly decreased the values to 9.75 ± 1.64 MEG/HPF, 15.0 mg/kg per d CsA to 9.66 ± 3.38 MEG/HPF, 3.2 mg/kg per d RAPA to 10.83 ± 1.60 MEG/HPF, and 6.4 mg/kg per d RAPA to 9.33 ± 1.75 MEG/HPF (all, P < 0.0001; Figure 7B). A greater effect was produced by CsA/RAPA combinations: 7.5/1.2 mg/kg per d to 9.83 ± 0.75 MEG/HPF and 15.0/3.2 mg/kg per d to 7.0 ± 0.89 MEG/HPF. The median effect analysis showed a range of interactions between CsA and RAPA spanning from synergistic to additive in nature, whether expressed as dose, whole-blood, or kidney tissue concentrations (Table 3). In contrast, the decrease in peripheral blood leukocyte counts associated with RAPA consistently showed an antagonistic interaction of concomitant CsA therapy. Overall, these findings suggest that addition of CsA significantly and probably synergistically exacerbates RAPA-induced myelosuppression.

Effect of CsA and RAPA alone or in Combination on Cholesterol-Containing Lipoprotein Fractions

RAPA monotherapy significantly increased the content of both low-density lipoprotein (LDL) cholesterol and serum cholesterol values in comparison with the modest increase induced by CsA alone. CsA/RAPA combination groups showed a significant interaction to increase LDL cholesterol (Figure 8A) but only modest effects on serum cholesterol (Figure 8B). These trends were confirmed by the median effect analysis (Table 2). In contrast, RAPA monotherapy caused a significant
dose-dependent increase in high-density lipoprotein cholesterol levels, whereas both CsA monotherapy and CsA-RAPA combination therapy produced only modest effects \( (P = 0.0002; \text{Figure 8C}) \). Thus, the property of RAPA that causes increased lipoprotein cholesterol levels seems to be augmented by a dynamic interaction with CsA.

**Discussion**

The present studies were designed to dissect the contributions of concentration-dependent pharmacokinetic interactions to the adverse effects associated with CsA or with RAPA treatment, namely renal dysfunction or bone marrow suppression and hypercholesterolemia, respectively. Whereas RAPA displayed nephrotoxic actions only at high doses, CsA almost uniformly produced concentration-dependent injuries—observations that were consistent with previous findings in animal models (25,41). A recent clinical trial reported better renal allograft function among patients that were treated with RAPA/Aza/Pred than those treated with CsA/Aza/Pred \( (42) \). However, because of the 40% acute rejection rate observed among patients that received the RAPA/Aza/Pred regimen, transplant physicians have been exploring the synergistic immunosuppressive effects of RAPA combined with CsA, even given that this regimen has been associated with augmented renal dysfunction \( (8,9) \).

We sought to compare the effects of ascending doses of each drug alone versus in combination in salt-depleted rats, an animal model that is not complicated by the occurrence of allograft rejection. We confirmed that CsA administration in this model caused functional and histopathologic renal changes similar to those observed in patients who undergo chronic drug administration \( (1,43) \). We also observed that salt-depleted rats that were treated with RAPA alone displayed reduced GFR values only at high doses, a finding that was consistent with the previous work of Andoh et al. \( (22) \) and DiJoseph et al. \( (24) \). Although the present findings of exaggerated reduction in renal function among salt-depleted rats that were treated with combinations of RAPA and CsA concur with the observations of Andoh et al. \( (22) \), Andoh et al. ascribed their findings to hyperglycemia, whereas we documented that it was due to potent pharmacokinetic interactions.

We demonstrated elsewhere that pharmacokinetic interactions potentiate the synergistic immunosuppressive effects of CsA and RAPA in rats \( (26) \) and that similar effects dramatically increase the whole-blood and, especially, the renal tissue concentrations of each agent, confirming and extending observations of Napoli et al. \( (27) \) in normal rats and those of Kaplan et al. \( (44) \) in renal transplant patients. The drug interaction may owe to mutual competitive interactions of both drugs as substrates for P-glycoprotein and for cytochrome P450 isozymes \( (45) \). The increases in concentration correlated with the observed alterations in SCr and GFR. Indeed, analysis of the adverse effects on the basis of concentrations rather than doses of CsA showed that the addition of RAPA actually mitigated the nephrotoxic injury, compared with that anticipated on the basis of the observed drug exposure. These findings suggest that the development of concentration-control algorithms may enable clinicians to minimize the renal injury associated with CsA/RAPA regimens. Indeed, it seems likely that the blinded design of the pivotal trials, which demanded the use of therapeutic CsA exposures in both the Aza and the placebo groups to protect the graft, may have led inadvertently to toxic CsA levels in the kidney transplants of the RAPA cohorts. Indeed, a logical extension of the present findings suggests that it may be more appropriate to combine doses of the non-nephrotoxic agent RAPA that are relatively higher than were administered in the pivotal trials with reduced doses of the nephrotoxic agent CsA, to exploit the synergistic immunosuppressive interactions and thereby minimize potentially toxic pharmacokinetic interactions.

Because RAPA inhibits transduction cascades evoked by a variety of cytokine signals, it may antagonize the nephrotoxicity of CNA by blocking the effects of a local humoral mediator such as angiotensin II \( (46) \). On the one hand, the production of both angiotensin II itself and its receptor is upregulated by CsA. On the other hand, angiotensin II recep-

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**Table 3. Median effect analysis of CsA/RAPA-induced bone marrow toxicity**

<table>
<thead>
<tr>
<th>Drug Measurement</th>
<th>Bone Marrow Cellularity</th>
<th>Megakaryocyte</th>
<th>White Blood Cell Count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dm/Cm  r CI Range</td>
<td>Dm/Cm  r CI Range</td>
<td>Dm/Cm  r CI Range</td>
</tr>
<tr>
<td>Dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>388.6 mg/kg 0.95</td>
<td>58.98 mg/kg 0.84</td>
<td>33.8 mg/kg 0.84</td>
</tr>
<tr>
<td>RAPA</td>
<td>97.0 mg/kg 0.91</td>
<td>21.99 mg/kg 0.93</td>
<td>0.3 mg/kg 0.97</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>—  0.09–0.82 (S)</td>
<td>1.44–0.11 (AD/S)</td>
<td>50–1.3 (AN)</td>
</tr>
<tr>
<td>Blood concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>30.6 ng/ml 0.93</td>
<td>11.22 ng/ml 0.83</td>
<td>6002.0 ng/ml 0.92</td>
</tr>
<tr>
<td>RAPA</td>
<td>639.9 ng/ml 0.65</td>
<td>115.0 ng/ml 0.85</td>
<td>1.38 ng/ml 0.97</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>—  0.21–0.97 (S)</td>
<td>1.2–0.26 (AD/S)</td>
<td>100–2 (AN)</td>
</tr>
<tr>
<td>Kidney concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>34.3 ng/g 0.73</td>
<td>8.4 ng/g 0.76</td>
<td>3509.0 ng/g 0.97</td>
</tr>
<tr>
<td>RAPA</td>
<td>3.4 ng/g 0.93</td>
<td>1.96 ng/g 0.93</td>
<td>217.0 ng/g 0.92</td>
</tr>
<tr>
<td>CsA/RAPA</td>
<td>—  0.32–0.89 (S)</td>
<td>1.3–0.33 (AD/S)</td>
<td>50–2 (AN)</td>
</tr>
</tbody>
</table>
tor–mediated signal transduction depends on p70s6 kinase, a product downstream from the action of mammalian target of rapamycin. Future studies to evaluate the content and activity of this cytokine and other humoral mediators will be critical to dissect the molecular basis of drug interactions in the kidney.

In contrast to the predominant role of pharmacokinetic interactions to produce renal dysfunction in animals that were treated with CsA/RAPA, there seemed to be more than additive effects of CsA to exacerbate the myelosuppressive and hypercholesterolemic toxicities produced by RAPA. The significant dose- and concentration-dependent myelosuppressive effects of RAPA were not shared by CsA. This toxicity presumably is related to the inhibitory effect of RAPA on the mammalian target of rapamycin, which catalyzes an essential step in the signal transduction pathway of proliferation-responsive cell lines upon addition of interleukin-3 (IL-3), IL-6, granulocyte colony stimulating factor (CSF), and granulocyte-macrophage CSF—all critical factors for hematopoiesis. The complementary actions of CsA to reduce production of at least some of these cytokines, including IL-3 and granulocyte-macrophage CSF, may potentiate the transduction blockade produced by RAPA. Although doses as high as 50 mg/kg per d RAPA had no effect on myelopoiesis in normal mice, Quesniaux et al. (47) observed impaired hemopoietic recovery during the 10-d period after injection of 5-fluorouracil. In humans, RAPA reduces the content of newly formed blood elements in a dose- and concentration-dependent manner, particularly during the early posttransplantation period, possibly because of the higher drug doses used during the induction phase and other associated perioperative stressors (48).

A similar pharmacodynamic interaction with CsA seemed to exacerbate hyperlipidemia, a well-recognized side effect of RAPA (49,50). In contrast to CsA, which seems to block LDL hepatic clearance mechanisms, RAPA displays a distinctive effect to reduce lipoprotein lipase activity in humans (Morrisett JD, Kahan BD, unpublished observations). Because the mammalian target of rapamycin is an intermediate in the pathway of insulin-driven release of lipoprotein lipase activity (Knutsen P, Kahan BD, unpublished observations), the increased LDL cholesterol may owe, at least in part, to dual sites of action of the two drugs.

On the basis of the present study, which shows that the effect of RAPA to potentiate drug-induced nephrotoxicity owes to increased CsA concentrations in both blood and kidney tissue—a pharmacokinetic interaction—clinicians must recognize that whole-blood levels do not reflect renal tissue levels. Indeed, these findings suggest that increasing RAPA exposure may permit substantial CsA reduction, thereby mitigating drug-induced nephrotoxic effects. In contrast, the effects of CsA to exacerbate RAPA-induced toxicities of myelosuppression and of hypercholesterolemia seem to be concentration-independent pharmacodynamic interactions, which were greater than those anticipated from the observed changes in whole-blood or tissue drug levels. Recent clinical experience suggests that drug doses should be adjusted across a therapeutic range of RAPA (and CsA) concentrations according to the patient’s proclivity to adverse effects: early posttransplantation

Figure 8. The effects of CsA and RAPA alone or in combination on lipid metabolism. Low-density lipoprotein cholesterol (LDL-ch) levels (A), total serum cholesterol (ch) levels (B), and high-density lipoprotein cholesterol (HDL-ch) levels (C) were measured in animals in the treatment groups described in the Figure 1 legend. (Coding for bars as indicated for legends to Figures 1 and 2.)
in the presence of impaired renal function, we now seek to achieve trough concentration ranges of RAPA = 10 to 20 ng/ml and CsA = 50 to 100 ng/ml. Alternatively, in the presence of myelosuppression or hyperlipidemia, we individualize drug doses to obtain concentration ranges of 5 to 15 ng/ml RAPA and 125 to 175 ng/ml CsA. Clearly, incisive molecular analyses of critical cytokine content/effects in experimental animals are urgently required to guide the formulation of insightful concentration-controlled, randomized clinical trials.

Acknowledgment

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References