NIH-PA Author Manuscript

Urine Metabolites Reflect Time-Dependent Effects of Cyclosporine and Sirolimus on Rat Kidney Function[*]

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Abstract

Background—The clinical use of the immunosuppressant calcineurin inhibitor cyclosporine is limited by its nephrotoxicity. This is enhanced when combined with the immunosuppressive mTOR inhibitor sirolimus. Nephrotoxicity of both drugs is not yet fully understood.

Methods—The goal was to gain more detailed mechanistic insights into the time-dependent effects of cyclosporine and sirolimus on the rat kidney by using a comprehensive approach including metabolic profiling in urine (^{1}H-NMR spectroscopy), kidney histology, kidney function parameters in plasma, measurement of glomerular filtration rates, the oxidative stress marker 15-F_{2t}-isoprostane in urine and immunosuppressant concentrations in blood and kidney. Male Wistar rats were treated with vehicle (controls), cyclosporine (10/25mg/kg/d) and/or sirolimus (1mg/kg/d) by oral gavage once daily for 6 and 28 days.

Results—Twenty-eight day treatment led to a decrease of glomerular filtration rates (cyclosporine -59%, sirolimus -25%). These were further decreased when both drugs were combined (-86%). Histology revealed tubular damage after treatment with cyclosporine, which was enhanced when sirolimus was added. No other part of the kidney was affected. ^{1}H-NMR spectroscopy analysis of urine (day 6) revealed time-dependent changes of 2-oxoglutarate, citrate and succinate concentrations. In combination with increased urine isoprostane concentrations these changes indicated oxidative stress. After 28 days of cyclosporine treatment, urine metabonomics shifted to patterns typical for proximal tubular damage with reduction of Krebs cycle intermediates and trimethylamine-N-oxide concentrations whereas acetate, lactate, trimethylamine and glucose concentrations increased. Again, sirolimus enhanced these negative effects.

Conclusions—Our results indicate that cyclosporine and/or sirolimus induce damage of the renal tubular system. This is reflected by urine metabolite patterns, which seem to be more sensitive than currently used clinical kidney function markers such as creatinine concentrations in serum. Metabolic profiling in urine may provide the basis for the development of toxicodynamic monitoring strategies for immunosuppressant nephrotoxicity.

Keywords

Cyclosporine; Sirolimus; nephrotoxicity; metabonomics; urine molecular markers

Introduction

Three decades ago, the introduction of the immunosuppressant calcineurin inhibitor cyclosporine revolutionized transplantation medicine and established transplantation as a feasible treatment option for patients with end-stage organ failure. Today calcineurin inhibitors remain a cornerstone of the prophylaxis against allograft rejection (1,2). Although in the past, due to their success in preventing rejection, toxicity of calcineurin inhibitors has largely been accepted. Attention has now increasingly focused on the fact that long-term use of calcineurin inhibitors such as cyclosporine is associated with a high risk of complications that can significantly compromise graft and patient survival (3). Acute and chronic toxicities include hypertension, diabetes, neurotoxicity (tremors and seizures) and renal dysfunction (4).

After kidney transplantation, calcineurin inhibitors contribute to chronic allograft nephropathy and have been identified as an independent risk factor and the major cause for late graft loss and increased patient mortality (5,6). Although the underlying molecular mechanisms are not yet completely understood, cyclosporine toxicity involves oxidative stress, apoptosis, metabolic changes (7,8) and an increase in vascular resistance resulting in decreased renal blood flow (9).

An attractive concept for reducing the prevalence of cyclosporine toxicity is its combination with other drugs that have additive or synergistic immunosuppressive activity and thus allow for lowering cyclosporine doses. In clinical trials, proliferation signal inhibitors such as sirolimus in combination with cyclosporine reduced the rate of acute rejection to less than 10%. This is despite markedly reduced exposures to both combination partners compared to the doses required for each agent alone to maintain clinical efficacy (10). The synergistic immunosuppressive activities of both drugs may be explained by the inhibition of subsequent steps in the activation and proliferation of T-lymphocytes. While cyclosporine blocks the progression of T-lymphocytes during the G_0 to G_1 transition of the cell cycle, sirolimus inhibits the mammalian target of rapamycin (mTOR), thereby blocking both the co-stimulation cascade, which augments interleukin-2 gene subscription during G_0 to G_1 progression, and the cytokine transduction pathway during the late G_1 phase (11). As shown in clinical trials, when used without cyclosporine, sirolimus seems to lack nephrotoxicity (12,13). However, there is some evidence that sirolimus may prolong kidney recovery and extend delayed graft function after ischemia/reperfusion injury (14,15) by a mechanism that involves enhanced necrosis, apoptosis and decreased proliferation of renal tubular cells (16).

Although combing sirolimus with cyclosporine significantly reduces the incidence of acute rejection, in the long term, sirolimus enhances the negative effects of cyclosporine on the kidney and increases creatinine serum concentrations (17-20). Since both drugs share pharmacokinetic pathways involving cytochrome P450 3A enzymes and are substrates of the same drug transporters such as p-glycoprotein, this effect can partially be explained by pharmacokinetic drug-drug interactions (19). In addition, there is evidence for toxicodynamic interactions (7).

Chronic immunosuppressant nephrotoxicity not only plays a key role in chronic nephropathy after kidney transplantation, it is also a major source for kidney dysfunction following transplantation of non-renal solid organs such as the liver, heart and small intestine (2). While acute immunosuppressant toxicity is usually associated with increased drug exposure and can reliably be detected, diagnosis of chronic toxicity is more challenging (21). The development of more sensitive diagnostic tools is warranted (22). The key to reduce or avoid the negative effects of chronic rejection and immunosuppressant toxicity is early detection (6). Once detected, damage by chronic rejection can be reduced by immunological intervention and, in the case of immunosuppressant toxicity, by modification of the immunosuppressive drug regimen. An attractive strategy is the identification of early metabolic changes in the transplant organ, blood or urine as molecular "signatures". In contrast to protein expression or histology changes, which take place over days or weeks, monitoring metabolic changes may allow for detection of an immediate response of immunological kidney damage and/or immunosuppressant toxicity (21,23,24). For the development of novel diagnostic strategies, a detailed understanding of the molecular mechanisms of the negative effects of cyclosporine alone and in combination with sirolimus is critical. The idea of using metabolic profiling to describe the effects of cyclosporine in the kidney is not new (23,25). The only other study that has evaluated the negative effect of immunosuppressants on urine metabolite patterns was focused on cyclosporine only and used high doses of 45mg/kg/d (25).

It is the goal of the present study to gain more detailed insights into the time-dependent effects of cyclosporine and sirolimus alone and in combination on the rat kidney and into the underlying molecular mechanisms by using a more comprehensive approach that included molecular, histological and pathological parameters indicative for kidney dysfunction. Also, based on our own pilot studies, we used cyclosporine and sirolimus doses that were known to result in blood concentrations close to those found in transplant patients after oral dosing. These doses were given over an extended time period of 28 days. The reason for this study design was to avoid the triggering of acute toxicity and cell death, and to characterize the effects of long-term exposure on the biochemistry of the kidney as reflected in urine. This dosing better simulates clinical reality, as well as avoiding confounding biochemical effects caused by salt depletion as is applied in most rat studies of immunosuppressant nephrotoxicity (26).

Since current clinical markers used to monitor nephrotoxicity such as serum creatinine fail to discriminate patients at risk early (6), metabolic profiling in urine may provide the basis for the development of toxicodynamic drug monitoring strategies. This could help to identify at-risk patients earlier and allow for kidney rescue therapies to commence before structural damage occurs (24). Such new strategies could facilitate improved individualization of immunosuppressive therapy.

Material and Methods

Animals

All animal protocols were approved by the University of Colorado Internal Animal Care and Use Committee in accordance with the National Institutes of Health guidelines (NIH publication No. 80-123). Ten- to fourteen-week-old male rats (Wistar Furth), weighing 280 to 330g, obtained from Charles River Labs (Wilmington, MA), were housed in a temperature and light controlled environment with access to tap water and food *ad libitum*. After at least two weeks of acclimatization, immunosuppressant treatment commenced.

Drugs

Commercial oral drinking solutions of cyclosporine (Neoral®, Novartis Pharma S.A., East Hanover, NJ) and sirolimus (Rapamune, Wyeth-Ayerst, Princeton, NJ) were administered by

daily oral gavage in a constant volume for 6 or 28 days, depending on group assignments. Sirolimus was administered in the unmodified formulation (1 mg/mL). Neoral® formulation was diluted in skim milk (1:10) to a final concentration of 10 mg/mL.

Experimental Groups

Sixty rats were randomly assigned to nine treatment groups (n=6/group):

(I)	vehicle controls	(skim milk for 6/28 days)
(II)	cyclosporine	10 mg/kg/day for 6 days
(III)	cyclosporine	25 mg/kg/day for 6 days
(IV)	sirolimus	1 mg/kg/d for 6 days
(V)	cyclosporine	10 mg/kg/day + sirolimus 1 mg/kg/day for 6 days
(VI)	cyclosporine	25 mg/kg/day + sirolimus 1 mg/kg/day for 6 days
(VII)	cyclosporine	10 mg/kg/day for 28 days
(VIII)	sirolimus	1 mg/kg/d for 28 days
(IX)	cyclosporine	10 mg/kg/day + sirolimus 1 mg/kg/day for 28 days

Experimental design

All drug doses were based on systematic dose finding studies during the development of the rat model, the goal of which was to achieve drug blood concentrations within limits that are reached in patients. Groups receiving twenty-five mg/kg/day cyclosporine were not included when long-term treatment effects were studied since a pilot study had shown that this dose in combination with 1 mg/kg/day sirolimus was associated with mortality of more than 50%.

Previous studies had shown that there still may be age-related differences in urine metabolite patterns in rats at the age used in the present study (27,28). To minimize an age-related bias, rats were randomly assigned to the treatment and control groups.

On day 5 or 27, rats were placed in metabolic cages for 24h-urine collections. On the final day (6 or 28), two hours after receiving the final drug doses, animals were prepared for clearance measurements as described below. Animals were sacrificed to collect kidneys for histology and measurement of tissue drug concentrations, and whole blood for the determination of sirolimus/cyclosporine concentrations. Plasma analysis for creatinine and blood urea nitrogen (BUN) and urine analysis for creatinine was performed by the University of Colorado Hospital Laboratory (Director: Dr. R. Lepoff) using validated standard methods.

Twenty-four hour urine collections always bear the potential risk of secondary bacterial contamination that may lead to "after-the-fact" changes of metabolite concentrations in urine samples. Urine was collected into sterilized containers. The collection method had been validated in a previous study excluding significant contamination demonstrating that NMR metabolite spectra were identical when urine was extracted immediately versus after storage in a sterilized collection container after 24 hours. The lack of bacterial contamination was further confirmed by bacteriological and biochemical methods.

Renal Function

Renal function was determined using the fluorescein isothiocyanate (FITC)-inuline method (29,30). Two hours after the final drug dosing, rats were placed on a thermostatically controlled surgical table and anesthetized by i.p. injection of ketamine (50mg/kg)/xylazine (10mg/kg)

(KetaVed™/TranquiVed™,Vedco Inc., St.Joseph, MO). A 10-0 silicone catheter was inserted into the jugular vein for maintenance infusion. After injecting 2mL of normal saline to provide sufficient intravasal volume, diluted (FITC)-inuline (Sigma, St. Louis, MO) (0.75mg/100mL saline) plus albumin (2.25 g/ 100 mL saline) were administered *via* perfusion pump for 2 hours at a rate of 2mL/h as previously described by Lorenz and Gruenstein (30). To monitor blood pressure throughout the experiment, a pressure transducer catheter (Millar Instruments, Houston, TX) was inserted into the carotid artery. After 1.5 hours of inuline infusion, a median laparotomy was performed, and a 10-0 silicone catheter was inserted into the left ureter. Urine was collected for 0.5 hours, and rats were sacrificed thereafter. Inuline concentration in plasma and urine was determined by fluorescence spectroscopy (Perspetive Biosystems Cytoflour Series 4000). GFR values (μl/min) were calculated using the formula (UxV/P,) were U equals inuline concentration in urine, V is urine output over time and P is inuline concentration in plasma. For a baseline correction, blank control plasma and urine samples were loaded with different concentrations of inuline and fluorescence absorption was obtained.

Drug concentration measurements

All drug concentrations were determined 4 hours after last dosing, when animals were sacrificed following renal clearance function measurements. For cyclosporine/sirolimus measurements, whole blood samples (500 μL) were collected in heparinized tubes. Flash-frozen renal tissue (100 to 200mg) was mortared in liquid nitrogen and homogenized with 2 mL KH_2PO_4 buffer (pH 7.4). For protein precipitation, 800 μL methanol and 0.2 mmol/L $ZnSO_4$ (80/20, v/v) were added to 200 μL of tissue/blood suspension. Cyclosporin D (250 μg/ L, Novartis Pharma AG, Basel, Switzerland) was added as an internal standard for cyclosporine derivates and ascomycin (Sigma-Aldrich, St. Louis, MO) as an internal standard for sirolimus (7). After centrifugation (13,000*g*, 5 min, 4°C), 100 μL of the supernatant was injected into the HPLC system onto the extraction column. Cyclosporine and sirolimus were quantified using a validated HPLC-MS assay (7).

Histology

For hematoxylin and eosin (H.E.) staining, kidney tissue samples were fixed in 10% buffered formaldehyde and embedded in paraffin, incubated for 5 minutes in Harris hematoxylin solution and for 60 seconds in eosin solution. Sections were washed with plain water, differentiated in 1% hydrochloric acid (HCl)+50% ethanol, and stain intensity was optimized in ammonia water. Finally, sections were rinsed in 70% ethyl alcohol and dehydrated in xylene solution.

Semi-quantitative scoring system

Evaluation of kidney histology was carried out in a blinded manner. Histologies were graded in regards to their tubular epithelial aspects, glomerular and vascular alterations in 10 to 15 randomly selected non-overlapping fields (x220) per rat on H.E. stains according to criteria published by Lombardi *et al.* (31). Tubular injury (TI) was graded (0 to 3) based on the presence dilation, atrophy, interstitional widening and presence of vacuolization. Glomerular injury (GI) was graded (0 to 3) for cellularity and capillary tuft collapse (percentage of glomeruli) as a marker for glomerular ischemia and damage. Renal arterioli were evaluated with respect to the presence of hyalinosis or thrombosis (0/1).

^{1}H NMR Spectroscopy

^{1}H-NMR urine analysis was performed using a Varian INOVA NMR 600MHz spectrometer equipped with a 5-mm HCN-PFG probe. Five hundred and fifty μL of urine were buffered with 73μL of 0.2mol/L potassium phosphate buffer in D_2O prior to analysis. The pH was finally adjusted to 5.65 - 5.75 with NaOD and DCl. To suppress water in urine, a standard Varian pre-

saturation sequence was used. ^{1}H-NMR spectra were obtained at 600 MHz using a spectral width of 12 ppm and 32K data arrays, and 64 scans with 90° flip angle. A D1 time of 14.8 seconds was used which was required to fully relax all protons in the samples including the TMSP (trimethylsilyl propionic-2,2,3,3,-d$_4$ acid dissolved in D$_2$O to 50mmol/L) protons. Analysis of NMR data was performed using the MesTreC software version 4.4.1.0 (MesTreLab Research, Coruna, Spain). Drift correction, zero filling from 32K to 64K data points and a Gaussian window function were applied to the FID prior to Fourier transformation. Spectra were referred to TMSP (0ppm) as an external standard. Prior to integration, all ^{1}H NMR spectra were manually corrected for phase and baseline distortions. Because of different overall urine concentrations, all spectra were normalized so that the total area of each urine spectrum reached the same value (32,33).

15-F$_{2t}$-Isoprostane (previous nomenclature: 8-iso-prostaglandin-F$_{2\alpha}$)

Urine samples were analyzed using a validated LC/LC-MS/MS method (34). The HPLC system consisted of Agilent series 1100 components (Santa Clara, CA) and was set up similar to that used for quantification of immunosuppressant concentrations (7). It was interfaced with a triple quadrupole mass spectrometer (API5000, Applied Biosystems, Foster City, CA) run in negative multiple reaction monitoring (MRM) mode. Peak area ratios obtained from MRM mode of the mass transitions for 15-F$_{2t}$-isoprostane (m/z 353 → 193) and the internal standard 15-F$_{2t}$-isoprostane-d$_4$ (m/z 357 → 197) were used for quantification.

Statistical analysis

All numerical data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) method was used to determine group differences. Additional group comparison was performed using post-hoc Holm-Sidak analysis. The significance level was set at p<0.05 for all tests. As mentioned above, each experimental group consisted of six animals. Software used were SigmaPlot (version 9.01) and SigmaStat (version 3.11) both by Systat Software, Point Richmond, CA, USA.

Results

Physiological changes (Table 1)

Healthy control rats showed a weight gain of 7 ± 1% after 6 days. In contrast, rats treated with a combination of cyclosporine (10 or 25 mg/kg/day) and 1 mg/kg/day sirolimus for 6 days had a significant weight loss of -7 ± 3% and -11 ± 5%, respectively. The long-term combination treatment group that received 10 mg/kg/day cyclosporine plus 1 mg/kg/day sirolimus had a significantly lower weight gain (4 ± 7%) compared to untreated controls (27 ± 3% after 28 days) whereas a single treatment with either sirolimus or cyclosporine alone for 28 days had no negative effect on weight gain.

Serum creatinine and blood urea nitrogen concentrations (Table 1)

All cyclosporine-treated rats showed at least a trend towards higher serum creatinine concentrations compared to controls but there was no difference between days 6 and 28. Serum creatinine concentrations were significantly higher than in the vehicle control group when rats were treated with a combination of 25 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus for 6 days (1.8±0.9 versus control: 0.4±0.1mg/dL, p<0.001). Sirolimus alone did not significantly increase serum creatinine concentrations after 6 or 28 days. Changes observed in serum creatinine concentrations were accompanied by similar changes in blood urea nitrogen concentrations. Overall, the time-dependent differences between days 6 and 28 that were observed for fluorescein isothiocyanate inuline clearance, kidney histology and urine

metabolite patterns (*vide infra*) were not reflected by creatinine in serum or blood urea nitrogen concentrations.

Glomerular filtration rates (Figure 1)

Interestingly, compared to inuline clearance rates in controls ($358\pm132\mu L/min/100g$ body weight), sirolimus (1mg/kg/day) also slightly reduced glomerular filtration rates after 6 and 28 days. Cyclosporine treatment had a more significant effect than sirolimus, resulting in glomerular filtration rates of $96\pm37\mu L/min/100g$ body weight (10mg/kg/day) and $88\pm77\mu L/min/100g$ body weight (25mg/kg/day) after 6 days. The most severe reduction of inuline clearance was seen after long-term (28-day) treatment with a combination of 10 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus ($50\pm48\mu L/min/100g$ body weight).

Histology

After 6 days of treatment, none of the treatment groups revealed histological alterations to their kidney compared to vehicle-treated controls (results not shown). However, after 28 days of treatment, in the rats treated with cyclosporine, sirolimus or a combination of both, morphologic alterations were clearly seen that were mainly focused on the tubuli. Glomeruli and arterioli in these groups were again not different from control animals, and there was no evidence for necrosis or fibrosis.

After 28 days of combined cyclosporine/sirolimus treatment (Figure 2D), kidney cortex morphology displayed the most extensive changes seen as patchy alterations (inhomogeneous damage), which was the result of severe shrinking of the proximal tubular cytoplasm and atrophy plus luminal dilation of the distal tubular system in large parts (score 2+ to 3). Treatment with cyclosporine alone led to macro- and microvesicular tubular epithelial vacuolization (scores 2- to 2+) that was not seen in controls but had hardly any impact on tubular cell morphology (0 to 1) (Figure 2C). In rats treated with sirolimus alone, tubular damage was observed to a lesser degree than in the combined groups especially with a lesser degree of tubular cell vacuolization but with severe aspects of tubular epithelial atrophy (score 2+) (Figure 2B). In summary, the combination of 10 mg/kg cyclosporine and 1 mg/kg sirolimus for 28 days resulted in morphologic changes in the kidney that were the most severe with its main emphasis on the tubular system. In comparison, cyclosporine alone mainly induced tubular vacuolization and the main effect of sirolimus treatment was tubular atrophy.

Blood and tissue drug concentrations (Figure 3)

Although cyclosporine blood concentrations basically remained unaffected when cyclosporine and sirolimus were co-administered (Figure 3A), cyclosporine drug concentrations within the kidney were significantly increased after 6 days. This interaction was less pronounced after 28 days (Figure 3C). Co-administration of cyclosporine significantly increased sirolimus blood concentrations after 6 and 28 days (Figure 3B). Also, sirolimus tissue concentrations were several-fold higher in the presence than in the absence of cyclosporine:1 mg/kg/day sirolimus for 6 days: sirolimus kidney tissue concentration: 0.3 ± 0.1ng/mg wet weight, after 28 days: 0.1 ± 0.002ng/mg; sirolimus +10mg/kg/day cyclosporine: 4.3-fold increase of tissue sirolimus concentrations, sirolimus +25 mg/kg/day cyclosporine: 6.3-fold increase (all after 6 days); +10 mg/kg/day cyclosporine (28 days): 7-fold increase (Figure 3 D).

15-F_{2t}-isoprostane concentrations in urine (Figure 4)

Compared to untreated controls, 15-F_{2t}-isoprostanes showed a tendency of higher concentrations in urine (one-way ANOVA: p=0.054). Highest 15-F_{2t}-isoprostanes concentrations in urine (normalized based on creatinine concentrations) were observed after treatment with sirolimus alone and when sirolimus and cyclosporine were co-administered for

6 days (mean concentrations 6 to 10-fold higher than vehicle-treated controls). After 28 days, 15-F_{2t}-isoprostanes concentrations tended to be lower than in corresponding treatment groups after 6 days.

Metabolite patterns in urine as assessed by ^{1}H NMR spectroscopy (Figures 5-8)

Following 6 days of treatment, changes in the biochemical profiles of urine were generally less pronounced and exhibited higher standard deviations than after the 28-day treatment. Changes observed in urine metabolite ^{1}H NMR spectra compared to controls after 6 days of treatment were reduced concentrations of citrate, succinate and 2-oxoglutarate (only statistically significant when 10 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus were co-administered) and increased concentrations of lactate, acetate, creatine, trimethylamine (TMA), and hippurate (Figure 5). Also, concentrations of trimethylamine-N-oxide (TMAO) in urine were found to be elevated in the groups treated with sirolimus for 6 days, but not in those rats receiving cyclosporine alone. Treatment with cyclosporine or cyclosporine in combination with sirolimus displayed not only more extensive negative effects on the kidney after 28 than after 6 days but also time-dependent differences in the metabolite patterns. Compared to untreated controls, concentrations of glucose (control =sirolimus <cyclosporine <<cyclosporine +sirolimus) and lactate (control <sirolimus <cyclosporine <<cyclosporine+sirolimus) significantly increased (Figures 6 and 7). The same applied for acetate concentrations (control <<sirolimus =cyclosporine =cyclosporine+ sirolimus) (Figure 6). Creatinine, hippurate, as well as key metabolites of the Krebs cycle including citrate and 2-oxoglutarate (2-OG), were significantly decreased (creatinine: control =sirolimus =cyclosporine >>cyclosporine +sirolimus; hippurate: control >>sirolimus =cyclosporine =cyclosporine+ sirolimus; citrate: control >sirolimus >cyclosporine >>cyclosporine+ sirolimus; 2-oxoglutarate: control =cyclosporine >sirolimus =cyclosporine+ sirolimus) (Figure 6). TMAO significantly decreased (control >>sirolimus =cyclosporine =cyclosporine+ sirolimus) whereas TMA increased in all groups compared to the controls (control <cyclosporine <cyclosporine+ sirolimus <<sirolimus) (Figure 7). Changes in succinate concentrations did not change with statistical significance but there was a trend towards lower concentrations in rats treated with sirolimus and cyclosporine+ sirolimus compared to the controls (Figure 6). Figures 8 and 9 show representative series of ^{1}H NMR spectra (day 28) from the urine of treated and control animals. It must be noted that these spectra show ethanol signals, however, only in the animals that received cyclosporine and/or sirolimus and not in the control animals that received only skim milk. The drug formulations are the most probable source. The presence of ethanol in the drug formulations was confirmed by analysis of diluted samples of the formulations using NMR spectroscopy. As mentioned above, bacterial contamination is another potential source of alcohols that cannot completely be excluded. The fact that ethanol was only detected in spectra of animals that received the study drugs and in none of the animals that received skim milk only and that urine from treated animals and controls was always collected under the same conditions and at the same time makes drug formulations the more likely source.

Discussion

The goal of the present study was to assess the time-dependent effects of cyclosporine and its combination with sirolimus on the kidney and to evaluate the underlying molecular mechanisms. In previous animal studies on immunosuppressant nephrotoxicity, it has been well established that rat kidneys are less sensitive to the negative effects of cyclosporine than humans (35). Two major strategies have been used in the past to circumvent this problem; one is the use of high cyclosporine doses (25), the second is based on the so-called salt-depleted rat model (19,20,36), originally developed and described by Elzinga et al (37). Although the salt-depleted rat model represents a reproducible setting for cyclosporine toxicity, as of yet, the exact biochemical mechanisms remain unknown, and, as in cases of using supra-clinical

doses, it remains unclear how valid extrapolation of such data to transplant patients really is. It seems reasonable to expect that a model using normally fed rats that are exposed to doses of cyclosporine and other immunosuppressants resulting in blood concentrations close to the clinically relevant range over a sufficient period of time will better transfer to the clinical situation and increase the impact of pathophysiological, histological and biochemical changes found. Vice versa, in less physiologic models that use salt-depletion or supra-clinical doses to accelerate the development of immunosuppressant nephrotoxicity, results may be of limited use. Our experiments show that treatment of rats with cyclosporine for 28 days with or without the addition of sirolimus is sufficient to result in a detectable impairment of renal function (demonstrated by a significant reduction of glomerular filtration rates) and histomorphologic alterations (tubular vacuolization, tubular epithelial damage) that are considered typical for cyclosporine toxicity (38).

Cyclosporine and sirolimus blood concentrations, as measured using a specific and validated LC-MS/MS assay, were close to those reported in kidney graft patients after 4 hours (39). Doses of 1 mg/kg/day sirolimus, depending on the absence or presence of cyclosporine, led to average blood concentrations between 3.2 ± 1.7 and 18.7 ± 7.2 ng/mL, respectively, after 4 hours (Figure 3B), which yielded trough blood concentrations (C_{24h}) most likely at the lower end of the target blood concentration range of transplant patients (usually 3-15 ng/mL). We also confirmed as already reported in clinical studies (40) and other animal models (17,41) that the negative effects of cyclosporine on kidney function are enhanced by co-administration of sirolimus.

After establishing the validity of our rat model, we characterized the corresponding metabolic changes in urine caused by immunosuppressants. The 6-day time-point was chosen to assess time-dependency of histological and biochemical changes and to assess as to whether changes in urine metabolite patterns allow for prediction of renal histological and pathophysiological changes seen after 28 days of treatment. The time-dependency of the urine metabolite pattern changes found was more extensive than could be expected based on the existing literature. In retrospect, it would have been of advantage to collect urine at additional time points between 6 and 28 days, to follow the time-dependent changes in more detail, and to exclude that other important changes caused by additional toxicodynamic processes that may have occurred between days 6 and 28 were missed. Our data provides the rationale for the design of a future study that takes advantage of our non-invasive bioanalytical strategy and studies the time-dependency of urine metabolite patterns in response to immunosuppressant exposure in greater detail.

Our previous studies suggested that the negative effect of cyclosporine and its enhancement by sirolimus are mediated by a decrease in mitochondrial energy metabolism, caused by an increase in reactive oxygen species (7). $15\text{-}F_{2t}$-isoprostanes are considered to be reliable and chemically stable *in vivo* markers for oxidative stress (42,43) and it reasonable to assume that their concentrations in urine are a valid surrogate marker for the amount of immunosuppressant-induced oxygen radical formation in the kidney. As shown in Figure 4, $15\text{-}F_{2t}$-isoprostane urine concentrations in groups treated for 6 days showed the following rank order: 10 mg/kg/day cyclosporine <25 mg/kg/day cyclosporine <1mg/kg/day sirolimus <10 mg/kg/day cyclosporine+1 mg/kg/day sirolimus <25 mg/kg/day cyclosporine+1 mg/kg/day sirolimus. These data match our previous findings that showed higher intracellular oxygen radical concentrations following sirolimus treatment than after cyclosporine exposure alone (7). After 6 days, urine $15\text{-}F_{2t}$-isoprostane concentrations in the cyclosporine treated groups paralleled the decrease of glomerular filtration rate in the absence of any histological damage. In the long-term treatment, although now structural and functional changes in the kidney increased, $15\text{-}F_{2t}$-isoprostane urine concentrations decreased but were still above controls.

Thus, these results indicate a possible involvement of free radicals, especially in the early course of immunosuppressant induced toxicity.

^{1}H NMR has been described as a valuable tool to detect changes in urine metabolites (44-46) that could be correlated with specific histopathologic changes induced by certain nephrotoxins like hexachlorbutadiene (HCBD) (47) or S-(1,2-dichlorovinyl)-L-homocysteine (DCVHC) (48). In these animal studies, proximal tubular injury was associated with increased urine concentrations of glucose, lactate, hydroxybutyrate and decreased concentrations of hippurate, creatinine, succinate and citrate. Medullary injury including papillary necrosis was associated with an increase in TMAO, TMA, actetate and succinate (44,45,49,50). TMA, a volatile substance synthesized from choline in the gut before absorption into the blood system, is metabolized by flavin-containing monooxygenase into TMAO (50,51).

In an earlier study on patients with kidney transplantation, urine analysis by ^{1}H NMR also revealed increased levels of acetate, succinate, glycine, alanine, dimethylamine (DMA) and TMAO in patients with delayed or non-function of the graft. Since only the differences of TMAO were altered significantly, the authors concluded that this alone could serve as a future marker for post-transplant dysfunction (52), a statement that has to be questioned now, considering our data and the aforementioned results of other studies. It is reasonable to expect that alterations of urine metabolite patterns as molecular markers of renal damage are not specific.

In a previous study, rats were treated with 45mg/kg/day cyclosporine, which resulted in increased urine concentrations of glucose, acetate, TMA and succinate on day 7 (25). These results are consistent with our findings after 6 days. On the other hand, changes in urine metabolite patterns observed after 28 days of treatment with cyclosporine or a combination of cyclosporine and sirolimus in our study, match those described for nephrotoxins causing (*pars recta*) tubular injury (47,48). This corresponds to the histology results that also showed specific tubular damage.

Overall, our data indicated that the extent of changes in urine metabolic patterns reflects the severity of kidney damage as evaluated by histology and reduction in glomerular filtration rates. Transporters for glucose, lactate and Krebs cycle intermediates have been identified in the brush border membrane of the tubuli (53). Krebs cycle intermediates imported from urine are used as energy substrates by proximal tubular cells (54). After 28 days of treatment, cyclosporine alone and, to a larger extent, in combination with sirolimus increased lactate and glucose concentrations in urine and decrease the concentrations of Krebs cycle intermediates (Figure 6). As a possible mechanism, this suggests that the proximal tubulus cells cannot utilize glucose and lactate as energy substrates as usual and have to compensate by importing more Krebs cycle intermediates from the urine. Krebs cycle intermediates are imported from urine by the sodium-dicarboxylate symporter NaDC-3 transporter that is mostly located in the proximal tubulus cells in the kidney (55). Our results provide additional evidence that any changes in urine metabolite patterns are mainly induced by the negative effects of cyclosporine with and without co-administration of sirolimus on the proximal tubulus cell. If this hypothesis is true, changes in urine metabolite patterns observed in our rat study were mainly caused by active compensation of the negative effects of cyclosporine on the proximal tubulus cells.

Comparison of the urine metabolite patterns and isoprostane concentrations in urine showed that there is a time-dependent shift from primarily oxidative stress after 6 days of treatment to injury of the proximal tubuli after 28 days. This is not unexpected since cyclosporine toxicity is mediated by oxygen radical formation (7) and can be expected to precede tubular injury. Our results indicate that it will be important in future studies to include several time points in urine

metabolic profiling studies since the metabolite patterns differ depending on the time-dependent stage of injury.

For the enhancement of cyclosporine toxicity by sirolimus, two potential mechanisms can be described: a toxicodynamic mechanism involving a synergistic inhibition of high-energy phosphate metabolism (7) and a toxicokinetic interaction leading to increased cyclosporine kidney tissue concentrations in the presence of sirolimus (19). Our study results support both mechanisms. Co-administration of sirolimus and cyclosporine enhances the distribution of both cyclosporine and sirolimus into the kidney tissue and adds to the negative effects of cyclosporine on cell metabolism as reflected in an increase of isoprostane concentrations in urine and changes in the urine metabolite pattern.

Although it is well recognized that sirolimus alone does not induce nephrotoxicity (12,13,56, 57), our study shows that the kidney is not completely inert against sirolimus. Despite the fact that sirolimus treatment does not increase serum creatinine concentrations, changes in kidney histology were observed after 28 days, which typically appeared as a dilatation and atrophy of the tubular system. Also, sirolimus treatment showed a trend towards a decrease of glomerular filtration rates (Figure 1), an increase of urine isoprostane concentrations and a significant alteration of urine metabolite pattern (Figures 5-9).

If our experimental results in rats translate into the clinical situation, our study will have important implications for the management of transplant patients treated with cyclosporine and/or sirolimus, since one of the major problems with the diagnosis of immunosuppressant toxicity is that the currently used diagnostic markers such as creatinine in serum and kidney histology are rather insensitive or impractical. There is a significant lag time between the onset of immunosuppressant toxicity and the time-point when these markers show changes that are considered clinically relevant (6). Our results provide a potential explanation for these phenomena. Serum creatinine is mainly a marker for glomerular dysfunction but it seems that during immunosuppressant treatment, damage of the tubular system precedes that of the glomeruli. We were able to demonstrate that in addition to creatinine in serum, the monitoring of metabolites in urine such as glucose, isoprostanes and/or Krebs cycle intermediates may confer critical information. It is reasonable to expect that monitoring these urine metabolites either as a single or a combinatorial marker has the potential to be more sensitive than creatinine concentrations in serum since these are directly affected by the toxicodynamic effects of cyclosporine and/or sirolimus while creatinine will start to change only after secondary effects such as inflammation and/or interstitial fibrosis has negatively effected glomerular function. A clinical trial to assess whether the results of the present study can be translated to transplant patients is currently in progress. However, a brief summary of the first clinical results of a single dose (5 mg/kg cyclosporine in its Neoral formulation), placebo-controlled, cross-over healthy volunteer study suggests that this may indeed be the case, and this has been recently described by our group in a review paper (22).

In summary, (A) we established a normal-salt rat model for immunosuppressant nephrotoxicity based on drug exposure similar to patients; (B) we showed that the biochemical signature of cyclosporine and sirolimus is time-dependent, with oxidative stress dominating early after-treatment initiation and tubular damage after long-term treatment; (C) our metabolic profiling data that was supported by kidney histologies suggested that the negative effects of cyclosporine and/or sirolimus are focused on the proximal tubuli while other parts of the kidney such as glomeruli were not affected after 28 days of treatment; (D) we showed that both toxicokinetic and -dynamic mechanisms may be the reason for the enhancement of the negative effects of cyclosporine on the kidney by sirolimus and, most importantly, (E) our data supports our hypothesis that the negative effects of cyclosporine and/or sirolimus on the kidney are reflected by changes in urine metabolite patterns that are closely connected to histological and

biochemical changes and seem to be more sensitive than monitoring serum creatinine and blood urea nitrogen concentrations.

Our study provides a first step towards translational studies to assess the potential value of urine isoprostane, glucose and Krebs cycle intermediate concentrations in urine to develop a toxicodynamic therapeutic drug monitoring strategy for transplant patients (24). It also generated hypotheses for further in-depth mechanistic studies to evaluate the reasons for the changes of urine metabolite patterns by cyclosporine and/or sirolimus. Our data suggest that the proximal tubulus cells and the NaDC3 transporter are of special interest.

Non-Standard Abbreviations

Rapa, Sirolimus

CsA, Cyclosporine

D_2O, Deuterooxide

DCI, Deuterochloride

FID, Free induction decay

H.E., Hematoxylin and eosin staining

HCN PFG, Hydrogen carbon nitrogen PF gradient

HPLC, High-performance liquid chromatography

MRM mode, Multi reaction monitoring mode

MS, Mass spectrometry

MS/MS, Tandem mass spectrometry

mTOR, Mammalian target of rapamycin

NaOD, Deuterated sodium hyrdroxide

NMR, Nuclear magnetic resonance spectroscopy

TMA, Trimethyl amine

TMAO, Trimethyl amine-N-oxide

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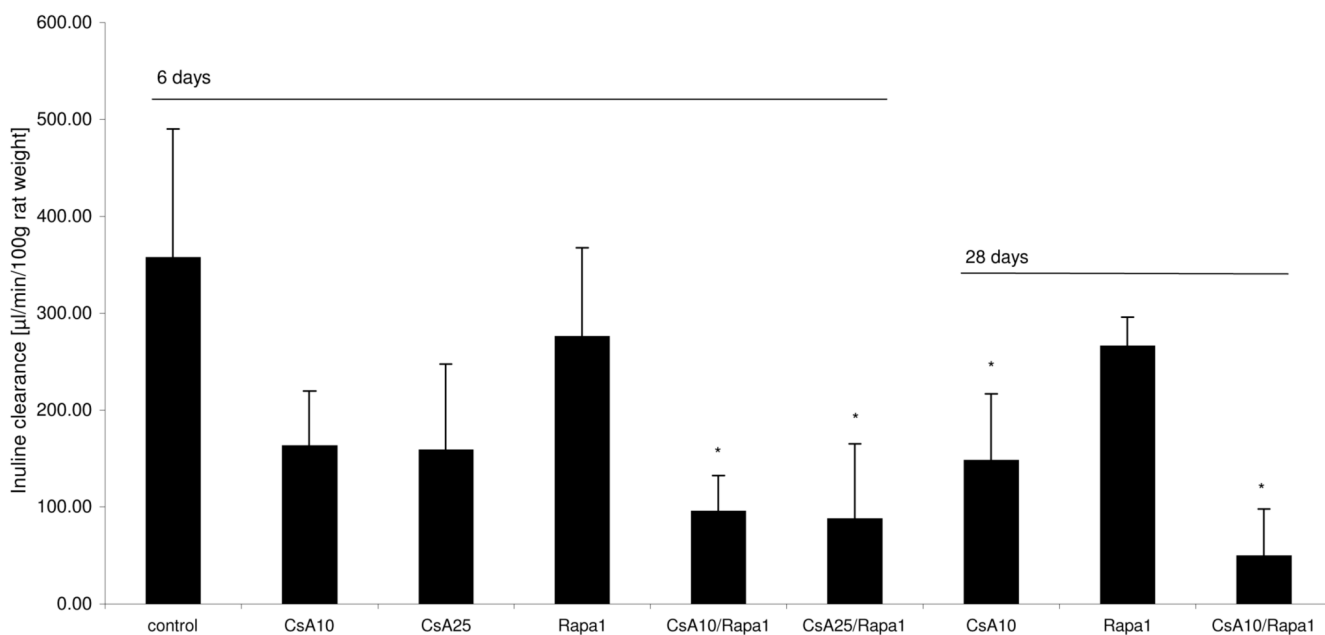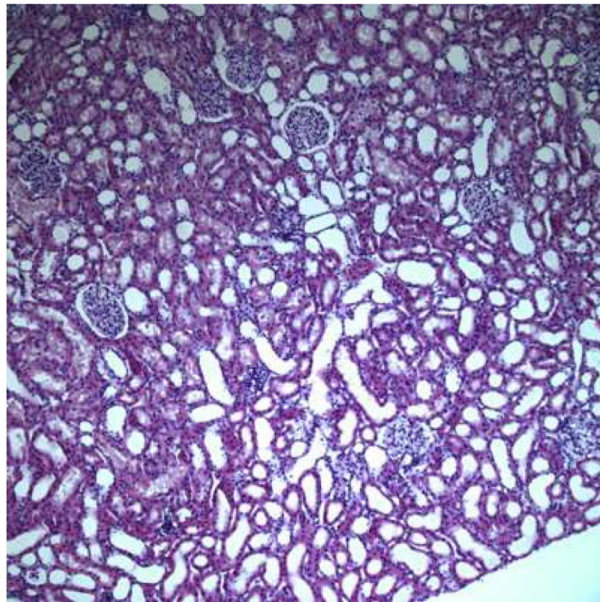
Figure 1.
Change in glomerular filtration rates (GFR) in the different treatment groups (all GFR values corrected for animal body weight and presented as means + standard deviations (n=6/group)), differences between groups was significant at p<0.001 (one-way ANOVA) *significant compared to untreated controls using a post-hoc pairwise multiple comparison (Holm-Sidak method).
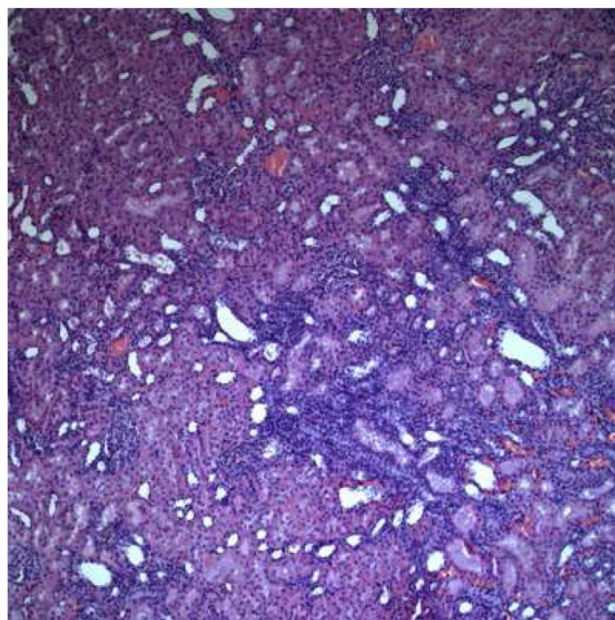Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus.
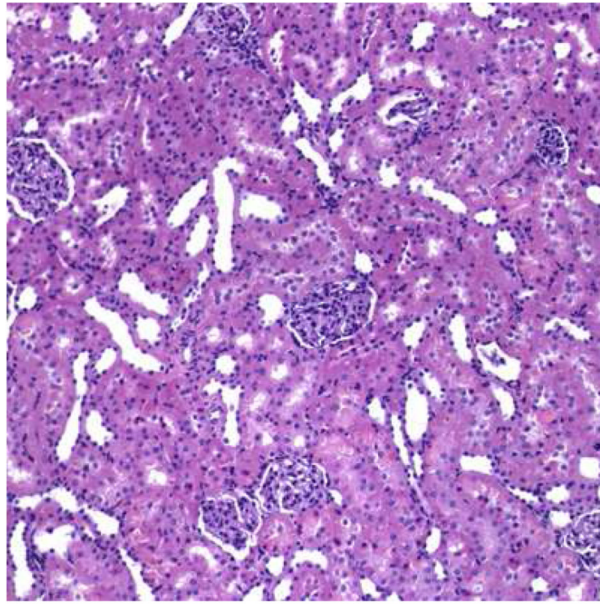
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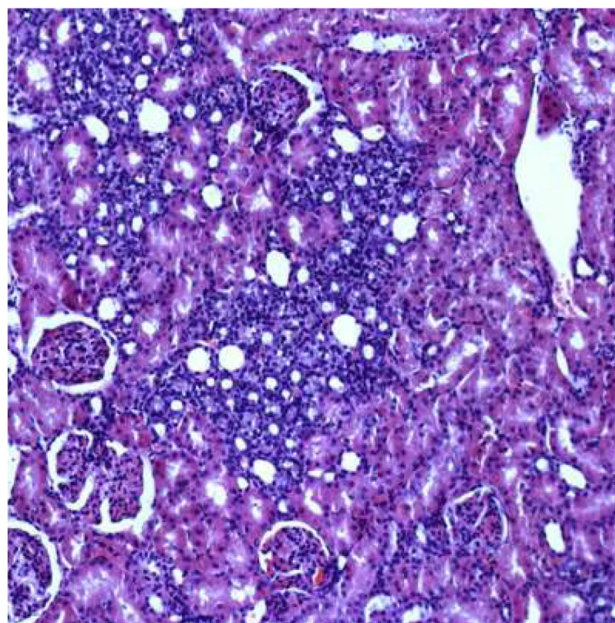
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Figure 2.
Representative histology (HE stain) of kidney tissues from the different treatment groups at day 28 (total number of tissue samples evaluated: n=6/ treatment group, all histologies (A-C): magnification 10×22, (D): magnification 20×22).
After 6 days of treatment, none of the groups revealed any significant histological changes in comparison to kidneys from vehicle-treated controls (A). After treatment with sirolimus (1 mg/ kg/day), kidneys revealed alterations of the tubular system appearing as severe atrophy and dilation. These changes were reproducible and clearly detectable in every animal (B). Following long-term treatment with cyclosporine (10 mg/kg/day), kidneys showed a patchy alteration caused again by mild tubular atrophy (shrinking of the proximal tubular cytoplasm)

plus atrophy and luminal dilation of the distal tubulus system. As another typical finding, there was a most significant presence of micro- and macrovesicular occlusion bodies in almost all tubular epithelial cells (C). These changes were similar and much more pronounced in the group receiving a combination treatment p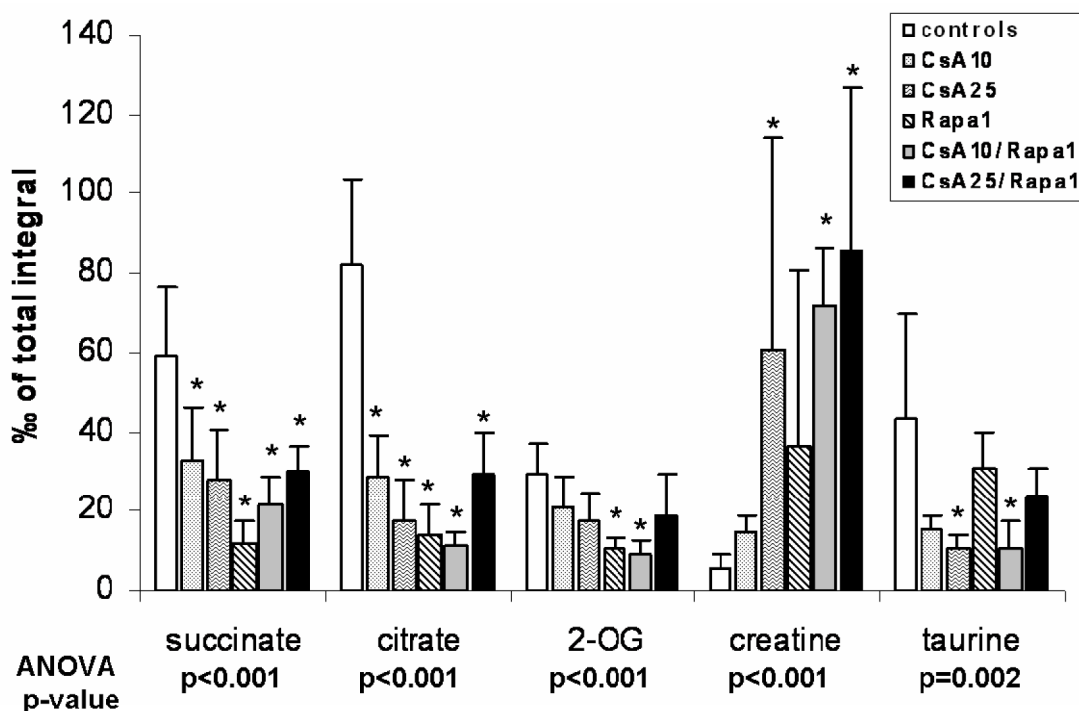rotocol of sirolimus and cyclosporine (D). Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/ day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/ kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/ kg/day cyclosporine and 1 mg/kg/day sirolimus.

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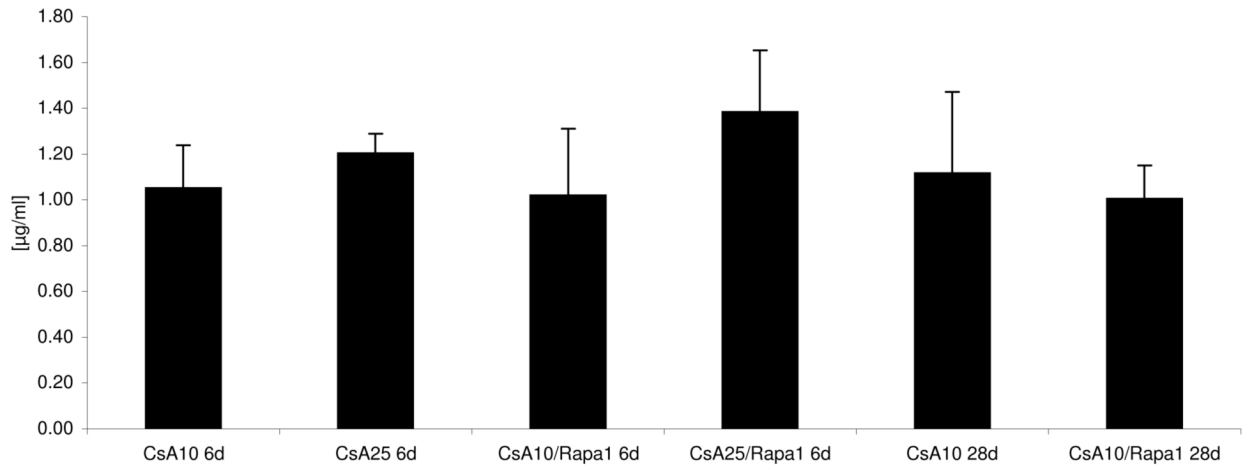
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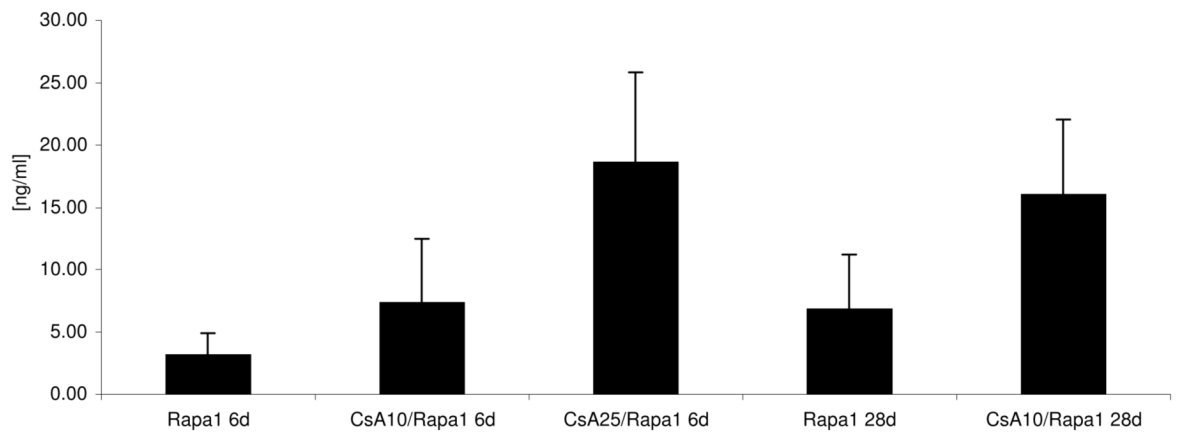
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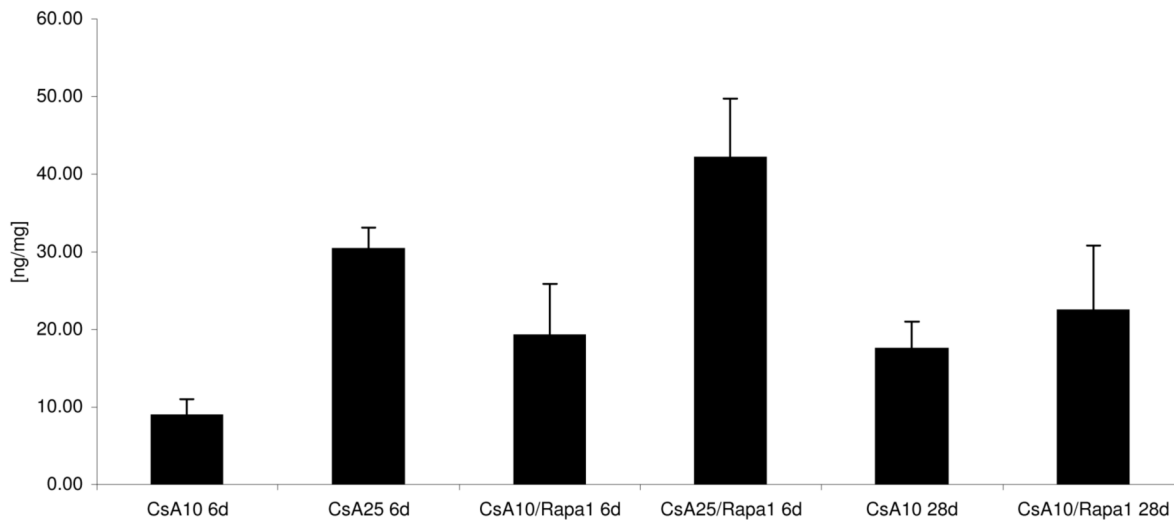
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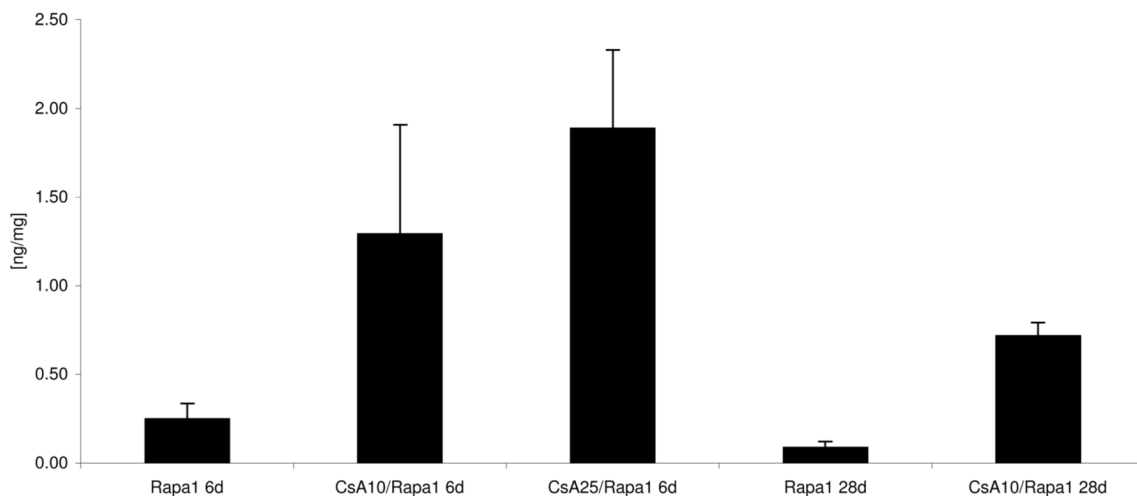
Figure 3. Blood and kidney tissue concentrations of cyclosporine and sirolimus 4 hours after the last dose

A) cyclosporine blood concentrations 4 hours after the last dose (all concentrations are presented as means + standard deviations (p=0.031); B) sirolimus blood concentrations 4 hours after the last dose (p<0.001); C) cyclosporine kidney tissue concentrations (p<0.001); D) sirolimus kidney tissue concentrations (p<0.001), all groups n=6, group comparison by one-way ANOVA
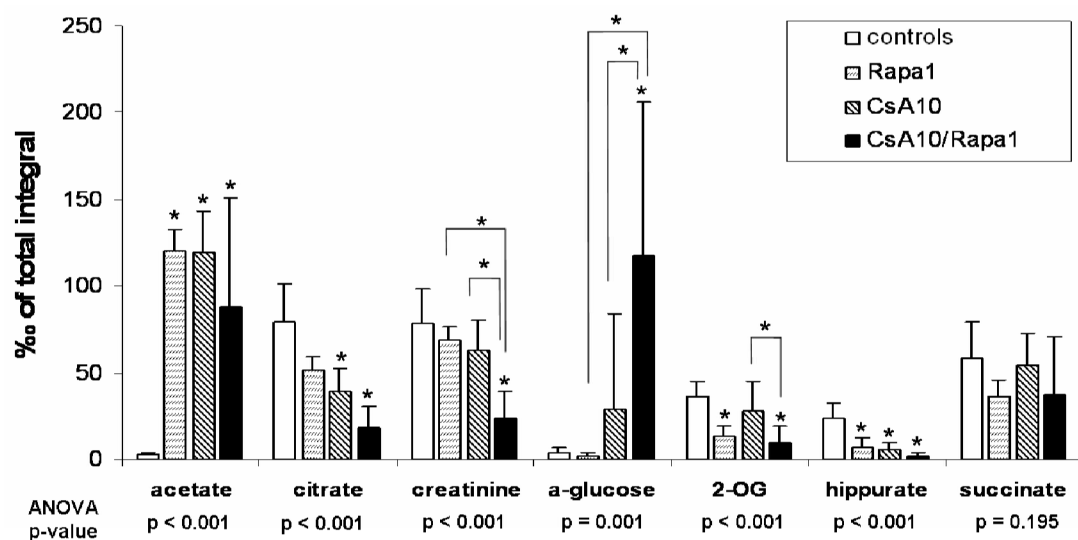
Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus.
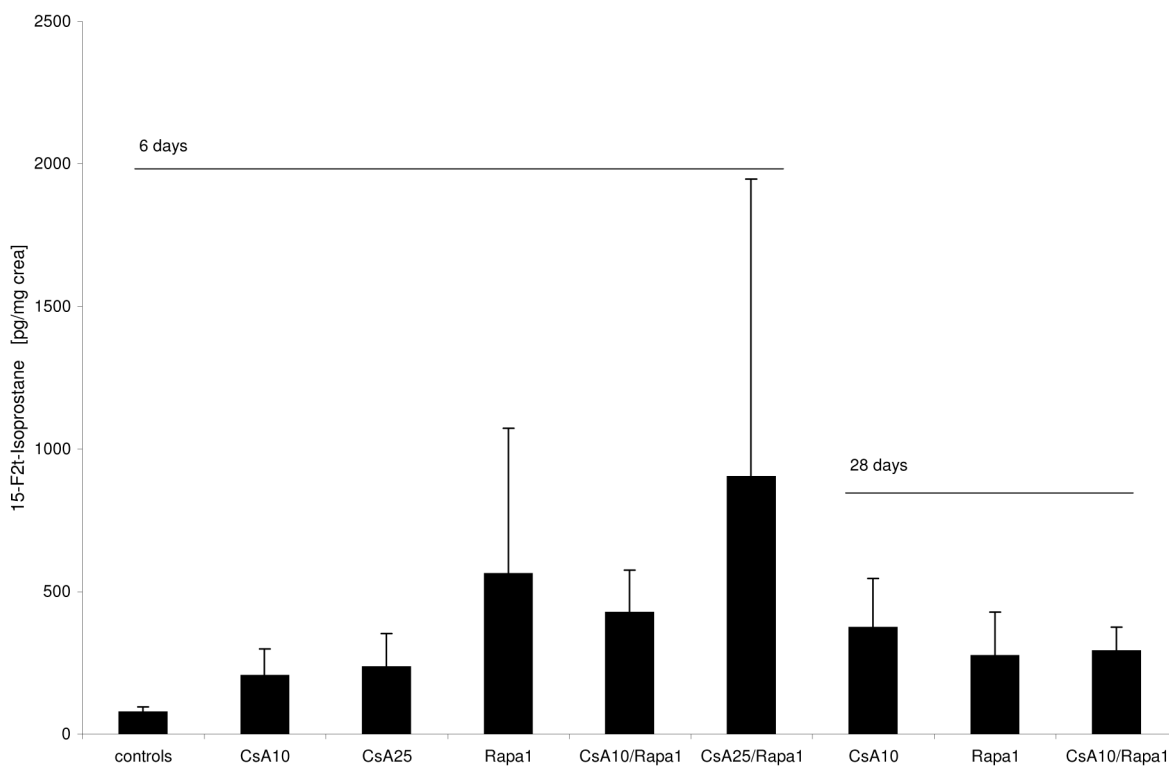
Figure 4. 15-F$_{2t}$-Isoprostane concentrations in urine after 6 and 28 days of treatment
All concentrations were normalized to urine creatinine concentration to compensate for
differences in urine concentrations and are shown as means + standard deviations (n=6 for all
groups). Group comparison (one-way ANOVA) failed to show statistically significant
differences (p=0.054).
Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/
day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/
kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/
kg/day cyclosporine and 1 mg/kg/day sirolimus.

Figure 5. Changes in urine metabolite patterns after 6 days of treatment
The pattern changes observed matched those typically associated with free radical formation
(58), (all urine metabolites determined semi-quantitatively by ^{1}H-NMR, all values were
normalized based on the total integral and are presented as means + standard deviations (n=6
for all groups), group comparison by one-way ANOVA, *significance levels estimated using
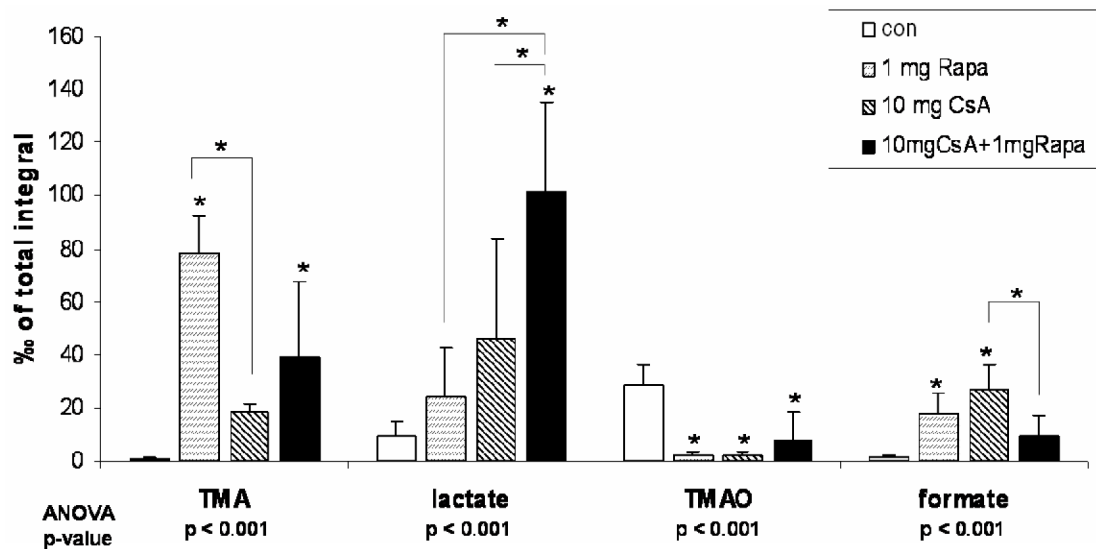a post-hoc pairwise multiple comparison (Holm-Sidak method).
Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/
day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/
kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/
kg/day cyclosporine and 1 mg/kg/day sirolimus.

Figure 6.
Changes in urine metabolite patterns after 28 days of treatment as assessed by ^{1}H-NMR spectroscopy- urine metabolites associated with S3 tubular damage (44), (all urine metabolites determined semi-quantitatively by ^{1}H-NMR, all values were normalized based on the total integral and are presented as means + standard deviations (n=6 for all groups), group comparison by one-way ANOVA, *significance levels estimated using a post-hoc pairwise multiple comparison (Holm-Sidak method).
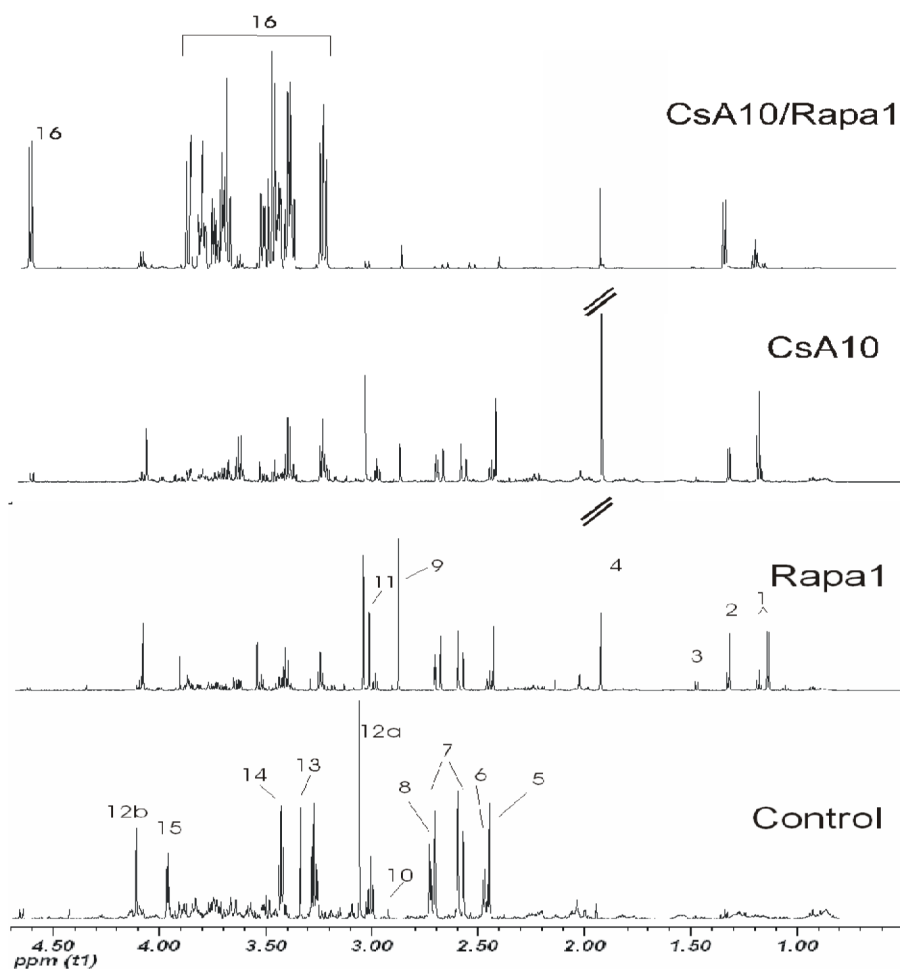
Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus.

Figure 7.
Changes in urine metabolite patterns after 28 days of treatment as assessed by ^{1}H-NMR spectroscopy (all urine metabolites determined semi-quantitatively by ^{1}H-NMR, all values were normalized based on the total integral and are presented as means + standard deviations (n=6 for all groups), group comparison by one-way ANOVA, *significance levels estimated using a post-hoc pairwise multiple comparison (Holm-Sidak method).
Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus.
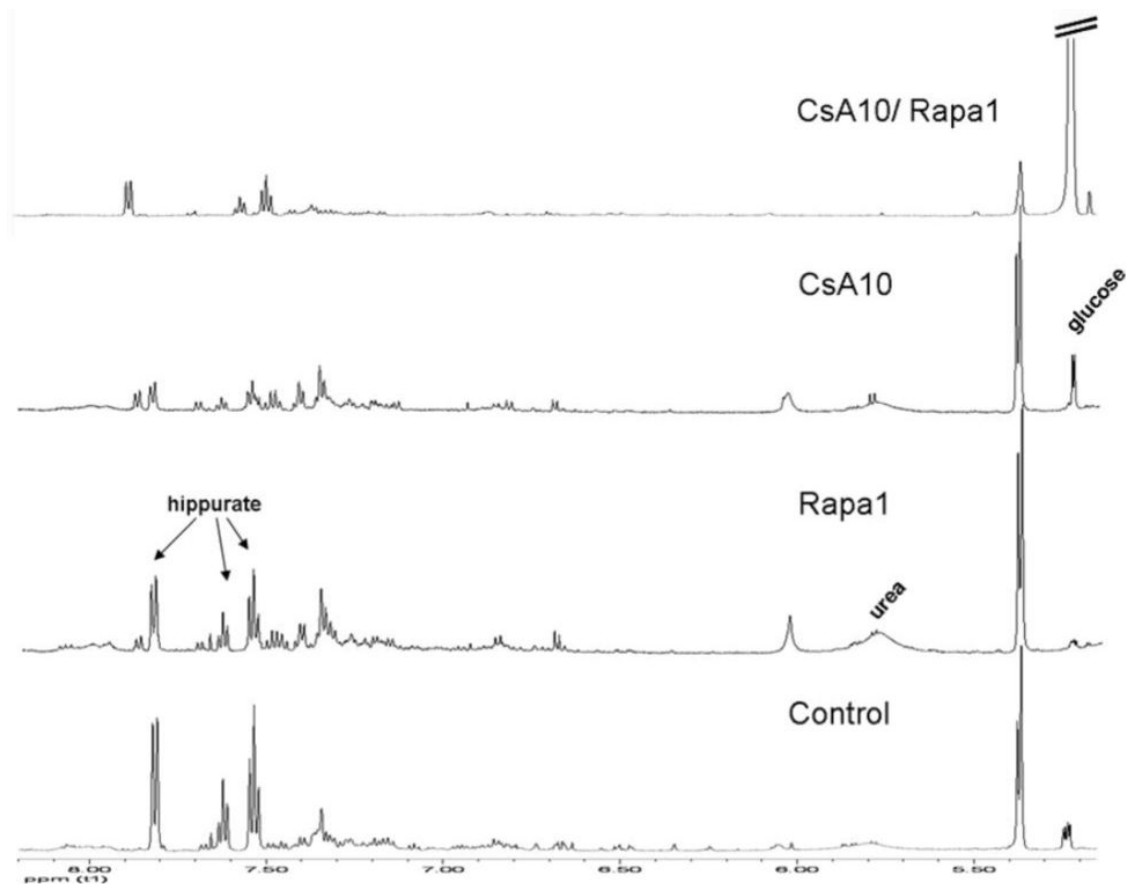
Figure 8. Representative ^{1}H-NMR spectra (aliphatic region) of urine extracts after treatment for 28 days

The total number of urine samples evaluated for each group was n=6. Signal assignments: 1 drug vehicle compounds, 2 lactate, 3 alanine, 4 acetate, 5 succinate, 6 2-oxoglutarate, 7 citrate, 8 dimethylamine, 9 trimethylamine, 10 dimethyl glycine, 11 creatine, 12a/b creatinine, 13 trimethylamine oxide, 14 taurine, 15 hippurate, 16 α-glucose.

Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/ day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/ kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/ kg/day cyclosporine and 1 mg/kg/day sirolimus.

Figure 9. Representative ^{1}H-NMR spectra (aromatic region) of urine extracts after treatment for 28 days

The total number of urine samples evaluated for each group was n=6. Glucose, urea and hippurate signals are shown.

Groups: con: vehicle-treated controls, CsA10: 10 mg/kg/day cyclosporine, CsA25: 25 mg/kg/day cyclosporine, Rapa1: 1 mg/kg/day sirolimus, CsA10/Rapa1: co-administration of 10 mg/kg/ day cyclosporine and 1 mg/kg/day sirolimus, CsA25/Rapa1: co-administration of 25 mg/kg/day cyclosporine and 1 mg/kg/day sirolimus.

Table 1

Changes in blood urea nitrogen, blood creatinine and body weight in the different treatment groups (all values are means ± standard deviations), n=6/group, one-way ANOVA among groups: p<0.001 for each parameter

		blood creatinine [mg/dL]	blood urea nitrogen [mg/dL]	body weight [% change over treatment time]
	control	0.4±0.1	17±3	7±1 (day6) 27±3 (day28)
6 days	CsA10	0.8±0.0	17±2	8±4
	CsA25	**1.0±0.1**[*]	25±5	**1±3**[*]
	Rapa1	0.4±0.1	16±4	9±5
	CsA10/Rapa1	0.8±0.5	**48±31**[*]	**-7±3**[*]
	CsA25/Rapa1	**1.8±0.9**[*]	**86±26**[*]	**-11±5**[*]
28 days	CsA10	0.7±0.1	34±6	**36±5**[*]
	Rap1	0.6±0.1	19±1	23±12
	CsA10/Rapa1	0.8±0.2	**42±13**[*]	**4±7**[*]

[*] significant compared to untreated controls using a post-hoc pairwise multiple comparison (Holm-Sidak method).