Sirolimus, but not the structurally related RAD (everolimus), enhances the negative effects of cyclosporine on mitochondrial metabolism in the rat brain

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1   Clinical studies have shown enhancement of cyclosporine toxicity when co-administered with the immunosuppressant sirolimus. We evaluated the biochemical mechanisms underlying the sirolimus/cyclosporine interaction on rat brain metabolism using magnetic resonance spectroscopy (MRS) and compared the effects of sirolimus with those of the structurally related RAD.

2   Two-week-old rats (25 g) were allocated to the following treatment groups (all $n = 6$): I. control, II. cyclosporine (10 mg kg^{-1} d^{-1}), III. sirolimus (3 mg kg^{-1} d^{-1}), IV. RAD (3 mg kg^{-1} d^{-1}), V. cyclosporine + sirolimus and VI. cyclosporine + RAD. Drugs were administered by oral gavage for 6 days. Twelve hours after the last dose, metabolic changes were assessed in brain tissue extracts using multinuclear MRS.

3   Cyclosporine significantly inhibited mitochondrial glucose metabolism (glutamate: $78 \pm 6\%$ of control; GABA: $67 \pm 12\%$; NAD$^+$: $76 \pm 3\%$; $P < 0.05$), but increased lactate production. Sirolimus and RAD inhibited cytosolic glucose metabolism *via* lactate production (sirolimus: $81 \pm 3\%$ of control, RAD: $69 \pm 2\%$; $P < 0.02$). Sirolimus enhanced cyclosporine-induced inhibition of mitochondrial glucose metabolism (glutamate: $60 \pm 4\%$; GABA: $59 \pm 8\%$; NAD$^+$: $45 \pm 5\%$; $P < 0.02$ versus cyclosporine alone). Lactate production was significantly reduced. In contrast, RAD antagonized the effects of cyclosporine (glutamate, GABA, and NAD$^+$, not significantly different from controls).

4   The results can partially be explained by pharmacokinetic interactions: co-administration increased the distribution of cyclosporine and sirolimus into brain tissue, while co-administration with RAD decreased cyclosporine brain tissue concentrations. In addition RAD, but not sirolimus, distributed into brain mitochondria.

5   The combination of cyclosporine/RAD compares favourably to cyclosporine/sirolimus in regards to their effects on brain high-energy metabolism and tissue distribution in the rat.
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Introduction

The calcineurin inhibitor cyclosporine (INN: ciclosporin, Figure 1) is used as an immunosuppressant after transplantation and for the treatment of immune diseases (Kahan, 1989; Faulds *et al.*, 1993). The clinical use of cyclosporine is limited by its toxicity in combination with its narrow therapeutic index (Kahan, 1989). Neurotoxicity is one of the most serious side effects and incidences

between 25 and 55% have been reported in transplant patients (Hauben, 1996; Gijtenbeek *et al.*, 1999). Since first reported by Calne *et al.* (1979), a wide range of cyclosporine-associated neurological symptoms have been described, ranging from mild symptoms such as tremor, agitation, insomnia, anxiety, amnesia, headache, and paraesthesia to severe neurotoxicity resulting in seizures, cortical blindness, aphasia, ataxia, and stroke-like episodes (Hauben, 1996; Gijtenbeek *et al.*, 1999). The incidence of cyclosporine neurotoxicity is increased in patients with high cyclosporine blood levels, but neurotoxicity also occurs

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A

cyclosporine

B

sirolimus

C

RAD
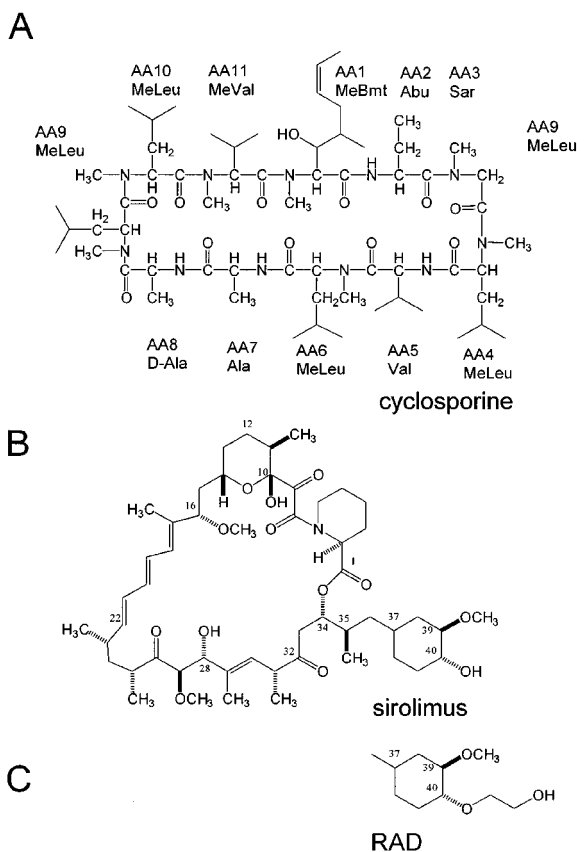
Figure 1 Structures of cyclosporine (A), sirolimus (B) and RAD (C). Numbering of the macrolide immunosuppressants sirolimus and RAD follows the IUPAC guidelines (IUPAC, 1993). (C) shows the region of the RAD molecule different from sirolimus.

centrations within the therapeutic target range (Hauben, 1996; Gijtenbeek *et al.*, 1999).

In *in vitro* studies, we showed that cyclosporine primarily inhibits mitochondrial energy production in the brain (Serkova *et al.*, 1996; 1999). Reduction of high-energy phosphate concentrations by cyclosporine was also found in kidney (Henke *et al.*, 1992; Massicot *et al.*, 1997), liver (Salducci *et al.*, 1996), and intestine (Madsen *et al.*, 1995; Gabe *et al.*, 1998).

Recently, the macrolide immunosuppressants sirolimus (rapamycin, Figure 1) and its 40-O-(2-hydroxyethyl) derivative RAD (INN: everolimus, Figure 1) have emerged as promising combination partners for cyclosporine. In the lymphocyte, sirolimus and RAD bind to FK-binding proteins and the immunophilin/sirolimus complex and probably also the immunophilin/RAD complex bind to mTOR, the mammalian target of rapamycin (Gummert *et al.*, 1999). Inhibition of mTOR arrests the interleukin-2 driven T-cell proliferation at the G_1- S-interface (Sehgal, 1998). Since the calcineurin inhibitor cyclosporine and sirolimus/RAD inhibit subsequent steps in the T-cell-mediated immune response, combination of cyclosporine and sirolimus or RAD results in synergistic immunosuppressive activity (Kahan, 1997; Stepkowski *et al.*, 1996; Schuurman *et al.*, 1997; Hausen *et al.*, 2000).

Cyclosporine and sirolimus/RAD have different toxicity spectra, with the only exception being hyperlipidemia (Brattström *et al.*, 1998). Although sirolimus by itself is not nephrotoxic, animal (Andoh *et al.*, 1996) and clinical studies (Kahan & The Rapamune US Study Group, 2000) have shown that sirolimus enhances cyclosporine nephrotoxicity. We showed in isolated, perfused rat brain slices that sirolimus and cyclosporine synergistically reduce high-energy phosphate concentrations (Serkova *et al.*, 1999). In the same *in vitro* model, RAD showed the opposite effect and antagonized the cyclosporine-mediated inhibition of mitochondrial oxidative phosphorylation (Serkova *et al.*, 2000a). However, these *in vitro* studies exclusively focused on high-energy phosphate metabolism. It is still unclear how sirolimus enhances the inhibition of oxidative phosphorylation by cyclosporine and why the structurally related RAD and sirolimus have opposite effects on high-energy metabolism in the presence of cyclosporine. *In vitro* (Serkova *et al.*, 1999; 2000a) potential pharmacokinetic and distribution interactions are ignored (Serkova *et al.*, 2000b). In addition, in these *in vitro* studies brain tissue was exposed to the study drugs for a maximum period of 2 h, a time period most likely too short to detect potential effects of the study drugs on protein expression.

Here, we treated rats with cyclosporine, sirolimus, and RAD as well as sirolimus or RAD in combination with cyclosporine for 6 days. The effects of the study drugs on brain metabolism were evaluated using multinuclear magnetic resonance spectroscopy (MRS). To assess pharmacokinetic interactions, concentrations of the study drugs in blood, brain tissue and brain mitochondria were quantified using h.p.l.c./ mass spectrometry. It was our goal to evaluate and compare the effects of sirolimus and RAD on brain cell metabolism in the presence of cyclosporine.

Methods

Materials

Cyclosporine and RAD were kindly provided by Novartis Pharma AG (Basel, Switzerland). Sirolimus was purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions (10 g l^{-1}) of each drug were prepared in absolute ethanol (Aldrich Chemicals, Milwaukee, WI, U.S.A.). Perchloric acid (PCA, 60%) as well as potassium hydroxide (KOH) for the extraction of brain tissue homogenates were from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Deuterated chemicals (D_2O, NaOD, and DCl) for MRS were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.) and NMR tubes of 5 mm diameter from Wilmad (Buena, NJ, U.S.A.). Cell metabolites in brain extracts were measured using a 500 MHz Bruker NMR spectrometer (Bruker Instruments Inc., Fremont, CA, U.S.A.). The system was controlled and data were processed using WINNMR software (Bruker Instruments Inc. Fremont, CA, U.S.A.). Methanol and zinc sulphate were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Percoll and other chemicals for mitochondria isolation were from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Concentrations of the study drug were measured using an h.p.l.c./h.p.l.c.-mass spectrometry (MS) system consisting of the following

components (all series 1100 h.p.l.c. components, Hewlett-Packard, Palo Alto, CA, U.S.A.): h.p.l.c. I: G1311A quaternary pump, G1322A degasser and G1329A autosampler equipped with a G1330A thermostat; h.p.l.c. II: G1312A binary pump, G1322A degasser, G1316A column thermostat and G1946A mass selective detector. The two h.p.l.c. systems were connected *via* a 7240 Rheodyne 6-port switching valve mounted on a step motor (Rheodyne, Cotati, CA, U.S.A.). The system was controlled and data were processed using ChemStation Software Revision A.06.01 (Hewlett-Packard, Palo Alto, CA, U.S.A.). A 10·2 mm extraction column (Keystone Scientific, Bellefonte, PA, U.S.A.) filled with Hypersil ODS-1 of 10 μm particle size (Shandon, Chadwick, U.K.) and a 50·4.6 mm analytical column filled with C_8 material of 3.5 μm particle size (Zorbax XDB C8, Hewlett-Packard, Palo Alto, CA, U.S.A.) were used. The internal standard cyclosporin D was a kind gift from Novartis (Basel, Switzerland) and 28,40-O-diacetyl rapamycin was synthesized and purified as described by Streit *et al*. (1996).

Animal studies

All animal protocols were reviewed and approved by the University of California, San Francisco, Committee on Animal Research.

The animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals', published by the National Institutes of Health (NIH publication No. 80-123, revised 1985).

Seventy-two Wistar rats (two weeks old, 28 ± 4 g body weight) were purchased from Charles River, Inc. (Wilmington, MA, U.S.A.) and were allocated to six treatment groups: (I) vehicle (control, $n = 12$); (II) 10 mg kg^{-1} cyclosporine (group Cs, $n = 12$); (III) 3 mg kg^{-1} sirolimus (group SRL, $n = 12$); (IV) 3 mg kg^{-1} RAD (group RAD, $n = 12$); (V) 10 mg kg^{-1} cyclosporine + 3 mg kg^{-1} sirolimus (group Cs + SRL, $n = 12$), (VI) 10 mg kg^{-1} cyclosporine + 3 mg kg^{-1} RAD (group Cs + RAD, $n = 12$). Stock solutions of the study drugs were dissolved in whole milk (1 : 10, final concentration 1 g l^{-1}). The study drugs were administered by oral gavage once daily. The final ethanol concentration was 10%. Animals in the control group were treated with 13 ml 10% ethanol/milk vehicle kg^{-1} body weight. This corresponded to the highest alcohol doses administered during the study (groups Cs + SRL and Cs + RAD). The doses were chosen on basis of a previous dose finding study (results not shown) with the goal to find oral doses of the study drugs resulting in brain tissue concentrations in the range of those that have previously been shown to have significant effects on the cell metabolism of isolated brain slices (Serkova *et al*., 1999; 2000a).

During our study, the animals were closely monitored, including daily evaluations of appearance, grooming, appetite, behaviour, activity and body weight. At the end of the 6-day study period and 12 h after the last study drug dose, the animals were decapitated and whole blood and brain tissues were collected. Blood samples were drawn into tubes with EDTA as anticoagulant. Tissues were immediately frozen in liquid nitrogen and stored at $-80°$C until PCA extraction ($n = 6$ per group) or h.p.l.c./h.p.l.c.-MS analysis ($n = 6$ per group). Samples were analysed within 20 days.

Measurement of brain cell metabolites using MRS

Frozen brain tissues were weighed; 2 g (wet weight) of each tissue sample was homogenized in a mortar grinder in the presence of liquid nitrogen and extracted with 6 ml ice-cold PCA (12%). The extracts were centrifuged, the aqueous phase was removed and neutralized using KOH. The samples were centrifuged once again and the aqueous phase was lyophilized overnight. The lyophilized brain PCA extracts were reconstituted in 400 ml deuterium oxide (D_2O) and adjusted to pH 7 using DCl and NaOD. The experiments with ^{1}H-, ^{13}C-, and ^{31}P-MRS were carried out as described previously (Serkova *et al*., 1996). Trimethylsilyl propionic-2,2,3,3,-d$_4$ acid (TSP) was used as an external standard for quantification in ^{1}H-MRS spectra. ^{1}H chemical shifts were referenced to TSP at 0 p.p.m. The use of an internal standard for ^{1}H-MRS allowed quantification based on areas under the ^{1}H-signals of single metabolites. The C3 peak of lactate at 21 p.p.m. was used as the chemical shift reference in ^{13}C-MRS. For ^{31}P-MRS, either phosphocreatine (PCr) at -2.33 p.p.m. or, if present, α-ATP at -9.90 p.p.m. were used as shift references. High-energy phosphate metabolism in the brain is a closed system, which means that a reduced concentration of one metabolite automatically leads to an increase of one or more other metabolites. Therefore, we calculated the relative concentrations of high-energy phosphates as the ratio of the ^{31}P-MRS peak area of a metabolite to the total area of all high-energy phosphate metabolites quantified.

Isolation of brain mitochondria

After treatment with the study drugs or their combinations for 6 days, the concentrations of cyclosporine, sirolimus, and RAD in rat brain mitochondria were determined using h.p.l.c./h.p.l.c.-MS analysis (*vide infra*). Brain mitochondria from rats treated with the vehicle or the study drugs were isolated by a modified procedure as described previously (Kristal & Dubinsky, 1997). Briefly: after decapitation, brains were rapidly dissected and placed in 10 ml ice-cold isolation buffer containing 350 mM sucrose, 2 mM HEPES and 1 mM EGTA (pH 7.4). After homogenization using a manual homogenizer, the brain suspension was centrifuged at 900 $\times g$ to remove cellular debris and *nuclei* for 10 min. The supernatant was decanted into a centrifuge tube and re-centrifuged under the same conditions. The supernatant was transferred into another centrifuge tube and was centrifuged at 10,000 $\times g$ for 10 min resulting in an olive-green mitochondrial precipitate. The mitochondrial fraction was homogenized in 1 part Percoll/7 parts buffer. The homogenate was layered over preformed discontinuous Percoll gradients consisting of a bottom layer of 40% Percoll in buffer and a top layer of 26% Percoll in buffer. The gradients were centrifuged at 31,000 $\times g$ for 45 min. The mitochondrial fraction, which was concentrated at the interface of the two layers, was collected, re-constituted in buffer, and centrifuged again. The supernatant was removed, and the pellet was reconstituted in 0.5 ml of 1 M KH$_2$PO$_4$ buffer pH 7.4. Mitochondria were extracted and analysed using h.p.l.c./h.p.l.c.-MS as described below.

*Measurement of tissue distribution of
immunosuppressants by h.p.l.c./h.p.l.c.-MS*

We used an h.p.l.c.-MS assay with automated online sample
extraction (h.p.l.c./h.p.l.c.-MS) for quantification of the study
drugs in blood, tissue and mitochondria, which was a
modification of the assay we described previously (Christians
et al., 2000). Brain tissues (1.5 g wet weight) were thawed and
weighed. Brain samples were homogenized with 2 ml
KH_2PO_4 buffer pH 7.4 (1 M) using an electric homogenizer.
For protein precipitation, 200 μl methanol/1 M $ZnSO_4$ (80/
20 v v^{-1}) was added to each 100 μl sample (homogenized
brain, blood or isolated mitochondria). Cyclosporin D and
28,40-O-diacetyl rapamycin were added as internal standards
for cyclosporine and sirolimus/RAD, respectively, resulting in
final concentrations of 100 μg l^{-1}. After centrifugation,
100 μl of the supernatant was injected onto the extraction
column. Samples were washed with a mobile phase of 40%
methanol and 60% 0.1% formic acid supplemented with
1 μmol l^{-1} sodium formate. The flow was 5 ml min^{-1} and the
temperature for the extraction column was set to 65°C. After
0.75 min, the switching valve was activated and the analytes
were eluted in the backflush mode from the extraction
column onto analytical column (flow 0.5 ml min^{-1}). The
mobile phase consisted of 90% methanol and 10% 0.1%
formic acid supplemented with 1 μmol l^{-1} sodium formate.
The mass spectrometer was run in the selected ion mode and
positive ions [M + Na]$^+$ were recorded. For all matrices, the
analytical recovery was >90%. In blood, brain and isolated
mitochondria, the assay was linear from 1 μg l^{-1} (lower limit
of quantification) to 500 μg l^{-1} for cyclosporine and
0.25 μg l^{-1} to 100 μg l^{-1} for the macrolides ($r^2 > 0.99$).

Data analysis

All results are reported as means±standard deviation (s.d.).
The results of the control and immunosuppressant-treated
groups were compared using unpaired Student's *t*-test or
analysis of variance in combination with Duncan grouping as
post hoc test (SPSS, version 9.0, SPSS Inc., Chicago, IL,
U.S.A.).

Results

Clinical monitoring

On study day 6, the average weight (±s.d.) of vehicle treated
animals in the control group was 166±12% of the initial
body weight (Figure 2). In contrast, cyclosporine-treated
animals (group Cs) showed no weight gain (91±10% of the
initial body weight). Animals treated with sirolimus or RAD
(groups SRL and RAD) were in better condition than the
cyclosporine-treated animals and gained weight, however,
slower than the control group (122±8 and 122±9%,
respectively). Co-administration of cyclosporine and sirolimus
had the most negative effect on the parameters recorded
including body weight (85±8% of the initial body weight). In
comparison to cyclosporine alone, animals treated with a
combination of cyclosporine and RAD lost slightly but
significantly less weight than those treated with cyclosporine
alone (97±13%). On day 6, body weights differed signifi-
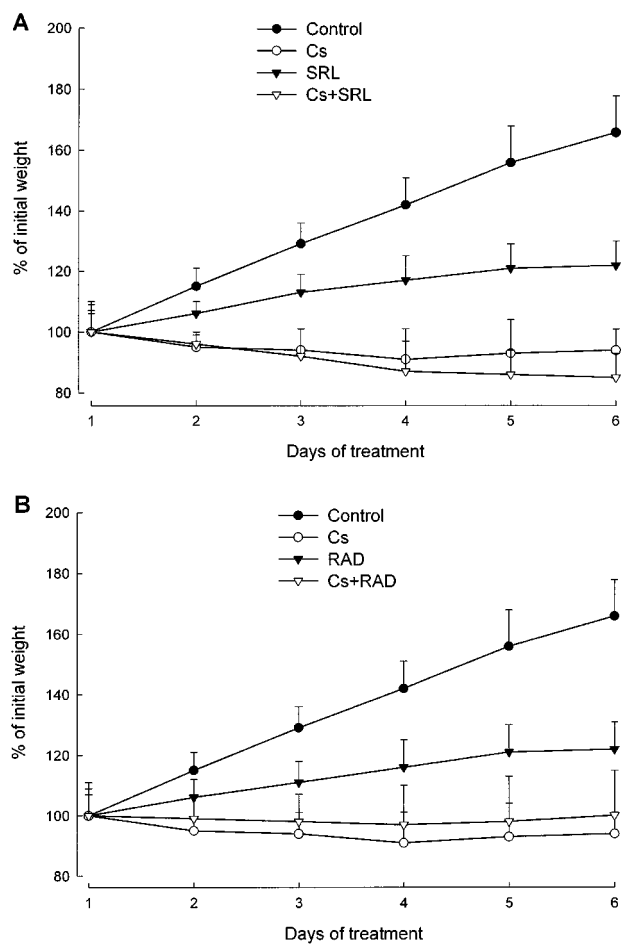
Figure 2 Effect of the study drugs and their combinations on weight
gain during the observation period. Data (mean±standard deviation,
$n = 12$) shows the per cent change at the beginning of each study day
in comparison with the weight at day 1 prior to dosing (=100%).
The study drugs were administered by oral gavage for 6 days. The
rats (2-weeks old, mean weight 28±4 g) received the following doses:
control, 13 ml vehicle (10% ethanol in milk); Cs, 10 mg kg^{-1} d^{-1}
cyclosporine; SRL, 3 mg kg^{-1} d^{-1} sirolimus; RAD, 3 mg kg^{-1} d^{-1}
RAD; Cs+SRL, 10 mg kg^{-1} d^{-1} cyclosporine+3 mg kg^{-1} d^{-1}
sirolimus; Cs+RAD, 10 mg kg^{-1} d^{-1} cyclosporine+3 mg kg^{-1} d^{-1}
RAD. (A) Comparison of groups Cs, SRL and Cs+SRL; (B)
comparison of groups Cs, RAD and Cs+RAD. The results of the
control group and Cs are shown in both (A) and (B) to facilitate
comparison.

cantly ($P < 0.001$) among groups as evaluated by analysis of
variance in combination with Duncan grouping: control
> RAD = SRL > Cs + RAD > Cs > Cs + SRL.

Effect of the study drugs on brain cell metabolism

^{1}H-MRS In control rats, the following cell metabolites
could be quantified: the neuronal marker N-acetyl aspartate
(NAA), the excitatory amino acid neurotransmitters gluta-
mate and aspartate, the inhibitory neurotransmitter γ-amino
butyric acid (GABA), the cellular osmolytes taurine and
myo-inositol as well as lactate (Table 1). In comparison to
the controls, cyclosporine (group Cs) decreased the brain
tissue concentrations of NAA (59% of control, $P < 0.002$,
Table 1) and those of the neurotransmitters glutamate (78%;

Table 1   The effects of the study drugs and their combinations on concentrations of neurotransmitters and cell metabolites in rat brain extracts analysed by ^{1}H-mrs

	Control (I) n = 7	Cs (II) n = 5	SRL (III) n = 5	RAD (IV) n = 5	Cs + SRL (V) n = 5	Cs + RAD (VI) n = 5
NAA	28.2 ± 4.1	16.5 ± 1.8***	21.6 ± 2.8*	20.6 ± 5.1*	14.2 ± 2.0***	22.2 ± 3.8
Aspartate	6.8 ± 2.0	4.9 ± 1.8	5.7 ± 1.3	5.5 ± 1.5	3.6 ± 1.4*	5.1 ± 0.6
GABA	6.7 ± 1.1	4.5 ± 0.9*	5.7 ± 1.6	5.8 ± 1.6	4.0 ± 0.6**	5.1 ± 1.6
Glutamate	15.0 ± 2.6	11.7 ± 1.5**	13.2 ± 2.5	13.4 ± 0.9	9.1 ± 1.3***	13.7 ± 0.6
Glutamine	8.3 ± 1.0	7.4 ± 1.9	10.8 ± 2.1*	11.1 ± 1.3**	9.7 ± 1.6	7.4 ± 2.0
Lactate	7.5 ± 0.8	10.4 ± 1.0***	6.1 ± 0.5**	5.2 ± 0.4***	4.0 ± 0.8***	6.5 ± 0.3*
Taurine	16.9 ± 4.2	16.8 ± 4.4	14.9 ± 1.1	15.3 ± 7.4	15.1 ± 4.2	15.4 ± 3.0
Myo-Inositol	10.9 ± 0.8	8.6 ± 2.2*	7.4 ± 1.1**	7.3 ± 0.9**	4.2 ± 0.7***	7.8 ± 1.0*

All concentrations (μmol g^{-1} wet weight) are means ± s.d. Data was analysed using analysis of variance in combination with Duncan grouping: *($P < 0.05$), **($P < 0.02$) and ***($P < 0.002$) in comparison to the control group. Abbreviations: Cs, cyclosporine; GABA, γ-aminobutyric acid; NAA, N-acetyl aspartate; SRL, sirolimus.

$P < 0.02$), and GABA (67%, $P < 0.05$). Lactate concentrations increased to 140% ($P < 0.002$). The effects of sirolimus (group SRL) and RAD (group RAD) were similar. In contrast to cyclosporine, the macrolide immunosuppressants decreased lactate concentrations (sirolimus: 81%, $P < 0.02$; RAD: 69%, $P < 0.002$). Sirolimus and RAD also slightly decreased the brain concentration of NAA and increased the concentrations of glutamine (Table 1).

Co-administration of cyclosporine and sirolimus (group Cs + SRL) had the most significant effect. Among the treatment groups, brain tissue extracts from animals in the Cs + SRL group contained the lowest concentrations of NAA (50% of control, $P < 0.002$), of glutamate (60%, $P < 0.002$), of GABA (59%, $P < 0.02$), and of lactate (53%, $P < 0.002$). In comparison to the controls, co-administration of cyclosporine and RAD, however, did not significantly affect most of the parameters measured with the exception of a slight decrease in lactate and myo-inositol concentrations (Table 1).

^{13}C-MRS   Although the natural abundance of ^{13}C-isotopes is only 1.1%, intensities of ^{13}C-MRS signals in our brain extracts were sufficient for reliable quantification of ^{13}C-groups of glucose and the following intermediates of the glucose pathways: aspartate, lactate, GABA, glutamate, glutamine, and oxaloacetate. In addition, the ^{13}C-signals of creatine, myo-inositol, NAA and taurine were detected. Representative ^{13}C-MRS spectra of rat brain PCA extracts from rats of the control, Cs, SRL and RAD groups are shown in Figure 3.

In comparison to the controls, brain extracts of cyclosporine treated animals showed reduced signal intensities of Krebs cycle-dependent metabolites such as glutamate, glutamine, GABA, and NAA (Figure 3). This reduction was accompanied by glucose (at 92.9 and 96.8 p.p.m.) and oxaloacetate (at 50 p.p.m.) signals above the detection limit, which were not present in the spectra of the controls. Additionally, the ^{13}C-signal of lactate, an important anaerobic glucose metabolite, was slightly increased. Glucose and oxaloacetate signals were undetectable in the ^{31}C-spectra of brain extracts from rats in the SRL and RAD groups. Lactate signals in these spectra were consistently smaller than in spectra of the control group. Characteristic changes after treatment with sirolimus or RAD were the significantly increased intensities of glutamine signals in comparison to the controls. Due to

the lack of an internal standard, absolute concentrations of metabolites based on the ^{13}C-MRS spectra could not be determined. We calculated the ratios of ^{13}C-signal intensities of Krebs cycle metabolites (aerobic glucose metabolism)/glycolysis intermediates (anaerobic glucose metabolism):

Krebs cycle/glycolysis (aerobic/anaerobic glucose metabolism) ratio =

$$\frac{\sum \text{glutamate} + \text{glutamine} + \text{GABA} + \text{aspartate} + \text{NAA} + \text{oxaloacetate}}{\sum \text{lactate}}$$

The ratios were 9.5 ± 1.1 for the control group ($n = 7$, mean ± s.d.), 5.6 ± 0.9 for the Cs group ($n = 5$), 14.1 ± 1.6 for the SRL group ($n = 5$) and 14.7 ± 2.9 for the RAD group ($n = 5$).

Co-administration of cyclosporine and sirolimus resulted in a reduction of all intermediates of glucose metabolism, from both Krebs cycle as well as from anaerobic glycolysis (Figure 4B) resulting in an aerobic/anaerobic glucose metabolite ratio of 13.0 ± 1.3. In comparison, glucose metabolism was less affected when cyclosporine and RAD were co-administered (ratio: 9.8 ± 1.4, Figure 4C). The ratios were significantly different with $P < 0.002$ (Duncan grouping, SRL = RAD = Cs + SRL > control = Cs + RAD).

^{31}P-MRS   The results of the ^{31}P-MRS analyses are summarized in Table 2. Treatment with cyclosporine reduced the relative concentrations of nucleoside triphosphates (NTP) to 34%, those of phosphocreatine to 30% and those of nicotinamide adenine dinucleotide (NAD$^+$) to 76% of the controls. In parallel, relative nucleoside diphosphate (NDP) concentrations were 2.4 fold and nucleoside monophosphate (NMP) concentrations 20.8 fold higher than in the control group. Sirolimus and RAD inhibited high-energy phosphate metabolism to a significantly smaller extent than cyclosporine (Table 2). Compared to the controls, sirolimus decreased NTP to 61% of the controls and increased relative NDP concentrations to 161% and NMP concentrations 5 fold. RAD decreased NTP concentration to 77% of controls, increased NDP concentrations to 171% but had no statistically significant effect on NMP concentrations. Both sirolimus and RAD did not significantly affect phosphocreatine or NAD$^+$ concentrations.

Again, co-administration of sirolimus and cyclosporine resulted in the most significant changes. At the end of the

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