

# Sirolimus/cyclosporine/tacrolimus interactions on bile flow and biliary excretion of immunosuppressants in a subchronic bile fistula rat model

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**1** The new immunosuppressive agent sirolimus generally is combined in transplant patients with cyclosporine and tacrolimus which both exhibit cholestatic effects. Nothing is known about possible cholestatic effects of these combinations which might be important for biliary excretion of endogenous compounds as well as of immunosuppressants.

**2** Rats were daily treated with sirolimus (1 mg kg<sup>-1</sup> p.o.), cyclosporine (10 mg kg<sup>-1</sup> i.p.), tacrolimus (1 mg kg<sup>-1</sup> i.p.), or a combination of sirolimus with cyclosporine or tacrolimus. After 14 days a bile fistula was installed to investigate the effects of the immunosuppressants and their combinations on bile flow and on biliary excretion of bile salts, cholesterol, and immunosuppressants.

**3** Cyclosporine as well as tacrolimus reduced bile flow (–22%; –18%), biliary excretion of bile salts (–15%; –36%) and cholesterol (–15%; –47%). Sirolimus decreased bile flow by 10%, but had no effect on cholesterol or bile salt excretion.

**4** Combination of sirolimus/cyclosporine decreased bile flow and biliary bile salt excretion to the same extent as cyclosporine alone, but led to a 2 fold increase of biliary cholesterol excretion. Combination of sirolimus/tacrolimus reduced bile flow only by 7.5% and did not change biliary bile salt and cholesterol excretion.

**5** Sirolimus enhanced blood concentrations of cyclosporine (+40%) and tacrolimus (+57%). Sirolimus blood concentration was increased by cyclosporine (+400%), but was not affected by tacrolimus.

**6** We conclude that a combination of sirolimus/tacrolimus could be the better alternative to the cotreatment of sirolimus/cyclosporine in cholestatic patients and in those facing difficulties in reaching therapeutic ranges of sirolimus blood concentration.

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**Keywords:** Cyclosporine; sirolimus; tacrolimus; cholestasis; rat

**Abbreviations:** Bsep, bile salt export pump; CyA, cyclosporine; CYP3A4, cytochrome P450 3A4; GSH, reduced glutathione; i.p., intraperitoneally; i.v., intravenously; LC, liquid chromatography; LC-ESI/MS, liquid chromatography-electrospray ionization/mass spectrometry; LDL, low-density lipoproteins; l.w., liver weight; Mrp2, multidrug resistance protein 2; Ntcp, Na<sup>+</sup>/taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; p.o., peroral; SRL, sirolimus; TRL, tacrolimus; VLDL, very low-density lipoproteins

## Introduction

Sirolimus (rapamycin, RAPAMUNE®) is a macrocyclic lactone isolated from *Streptomyces hygroscopicus*. Whereas cyclosporine (CyA) and tacrolimus (TRL), the two base therapy agents currently used in organ transplantation, achieve their effects principally by blocking calcineurin and thereby inhibiting interleukin-2 production, sirolimus (SRL) has no effect on calcineurin. Sirolimus reduces T-lymphocyte activation by inhibiting the interleukin-2-mediated signal transduction pathway (Seghal, 1995; Liu *et al.*, 1991). As the significant nephrotoxicity, neurotoxicity, and hypertension associated with CyA and TRL can be partly attributed to calcineurin blockade (Mayer *et al.*, 1997; Bennett *et al.*, 1996; Andoh *et al.*, 1997), SRL would be expected to have a

different toxicity profile. In preclinical studies, SRL has been shown to be as effective as CyA in maintaining survival of renal and cardiac allografts without causing nephrotoxicity (Granger *et al.*, 1995). When SRL was added to CyA-therapy in phase I (Murgia *et al.*, 1996) and phase II studies (Kahan *et al.*, 1999), no deleterious effects on renal function were observed, and the incidences of neurotoxicity and hypertension were unchanged. The most notable side-effects were hyperlipidaemia (both hypercholesterolaemia and hypertriglyceridaemia), thrombocytopenia, and leukopenia. The exact mechanisms of CyA- and SRL-induced hyperlipidaemia are not known yet. In the case of CyA, drug-induced cholestasis could be one important reason of hypercholesterolaemia, because CyA is known to cause cholestasis by inhibiting both basal and canalicular hepatocellular bile salt transporters (Stacey & Kotecka, 1988; Böhme *et al.*, 1994). In experiments with  $\alpha$ -naphthylisothiocyanate-treated mice, cholestasis was

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induced and increased levels of free cholesterol in the plasma were observed (Chisholm *et al.*, 1999). Because experimental transplantation studies suggest a synergism between SRL and TRL (Vu *et al.*, 1997), the combination of SRL/TRL is of increasing interest in clinical trials, too (Mcalister *et al.*, 2000). The first results of these studies indicate that the incidence of hypercholesterolaemia is lower in SRL/TRL-treated patients than in patients under SRL/CyA therapy. In studies with rats TRL was shown to reduce bile flow, as well (Sanchez-Campos *et al.*, 1998), but was less effective on cholestasis than CyA (Mizuta *et al.*, 1999). In a previous study in an acute bile fistula model in rats we could show that SRL/CyA reduced bile flow and biliary excretion of bile salts and cholesterol (Deters *et al.*, 2001). On the other hand, these parameters were not attenuated by SRL/TRL. To augment the clinical impact of these observations we investigated the effects of SRL, CyA, TRL, and the combination of either SRL/CyA or SRL/TRL on bile flow after treatment with these immunosuppressants in a sub-chronic rat bile fistula model over 2 weeks. Additionally, we measured the concentrations of these immunosuppressants and the main CyA metabolites in bile and blood to explore the role of biliary excretion of them for possible pharmacokinetic interactions that are of clinical importance, as well.

## Methods

### Animals

Male Wistar rats (300–400 g; breeder Medizinische Hochschule, Hannover Germany) were used throughout. The animal experiments were authorized by the ethical committee of the local government Hannover, Germany. The animals were treated in compliance with the indications of the Guide to the Care and Use of Experimental Animals, routinely used at our laboratory. During the time of immunosuppressant treatment they were housed in an individually ventilated cage system. They had free access to pellet feed (Altromin<sup>®</sup> standard diet, Lage, Germany) and tap water until surgery.

### Chemicals

Sirolimus (Rapamune<sup>®</sup>) was obtained from Wyeth-Pharma GmbH, Münster, Germany, as a solution (1 mg ml<sup>-1</sup>) for oral application. Thirty-two-desmethoxyrapamycin was kindly given by Wyeth-Ayerst, Princeton, U.S.A. Cyclosporine (Sandimmun<sup>®</sup>) as a solution (50 mg ml<sup>-1</sup>) for i.v. administration and tacrolimus (Prograf<sup>®</sup>) as a solution (5 mg ml<sup>-1</sup>) for i.v. administration were obtained by the local pharmacy. For intraperitoneal administration cyclosporine and tacrolimus were diluted 1/10 with 0.9% NaCl.

### Bile duct fistula model

Rats were divided into six groups (Table 1) and dosed daily for 14 days with SRL (1 mg kg<sup>-1</sup> p.o.), CyA (10 mg kg<sup>-1</sup> i.p.), TRL (1 mg kg<sup>-1</sup> i.p.), or a combination of SRL (1 mg kg<sup>-1</sup> p.o.) with either CyA (10 mg kg<sup>-1</sup> i.p.) or TRL (1 mg kg<sup>-1</sup> i.p.). After starving for 24 h the rats were anaesthetized with ethylether and blood was taken from the

animals by retro-orbital puncture for determination of bile salt, bilirubin, cholesterol, and triglyceride serum concentration just before the first application of the immunosuppressants. This procedure was repeated after 1 and 2 weeks of daily immunosuppressant treatment. Twelve hours after receiving the last dose of the immunosuppressants the rats were anaesthetized with urethane (6 ml kg<sup>-1</sup> 20% urethane solution  $\approx$  1.2 g kg<sup>-1</sup> intraperitoneally). The abdomen of the animals was opened by a central incision below the sternum. The common bile duct was prepared and cannulated with a PE 10 polyethylene tube just below the bifurcation of the common duct. After fixation of the fistula the abdomen was closed and the bile was collected every 15 min in Eppendorf vials. The whole experiment lasted for 90 min and was performed on a heated surgery table keeping the body temperature of the rats at 37°C. After 90 min whole blood was taken by heart puncture for determination of immunosuppressant blood concentrations and animals were killed by exsanguination.

**Analytical procedures** Bile flow was determined gravimetrically without correction for specific gravity and assuming a bile density of 1.0 g ml<sup>-1</sup>. The concentrations of triglycerides (Mcgowan *et al.*, 1983) and bilirubin in serum (Walters & Gerarde, 1970) and that of cholesterol (Siedel *et al.*, 1983) and bile salts (Mashige *et al.*, 1981) in bile and serum were determined by enzymatic colorimetric tests. Reagents for all colorimetric measures, which were performed on a DYNA-TECH MR5000, were purchased from Sigma-Aldrich (Deisenhofen, Germany). Biliary excretion of bile salts, cholesterol, and bilirubin were calculated as the concentrations of these parameters multiplied by bile flow. Bile salt-independent bile flow was obtained by calculating a correlation curve between bile flow versus biliary excretion of bile salts. The intercept with the ordinate (bile flow in the face of zero bile salt secretion) is an indirect measure for bile salt-independent bile flow (Erlinger, 1982). Cyclosporine and its main metabolites AM1, AM9, AM1c, and AM4N (Table 2) in blood and bile were measured by h.p.l.c as described (Christians *et al.*, 1988) with slight modifications. In brief, cyclosporine and its metabolites were extracted from blood by solid-liquid extraction and were quantified using cyclosporine D as internal standard. A liquid chromatograph 1090 Series II (Hewlett Packard, Waldbronn, Germany) was used as chromatographic system. One hundred  $\mu$ l of the extracted sample was injected into the chromatographic system. Acetonitrile and water with pH 3.0, adjusted with H<sub>2</sub>SO<sub>4</sub>, were used as eluents and the flow rate was 0.25 ml min<sup>-1</sup>. A precolumn (Hypersil C18, 5  $\mu$ , 10  $\times$  2 mm) from Schambeck SFD GmbH, Deisenhofen, Germany, and a reversed phase column (Hypersil C18, 5  $\mu$ , 250  $\times$  2 mm, phenomenex<sup>®</sup>, Aschaffenburg, Germany) were used and the column temperature was set to 67°C. Cyclosporine and its metabolites were eluted by a linear gradient. The proportion of acetonitrile/water was kept stable at 43/57 v v<sup>-1</sup> during the first 20 min of analysis. Then the ratio of acetonitrile/water was slowly enhanced from 20 to 35 min to 65/35 v v<sup>-1</sup> and from 35 to 45 min to 75/25 v v<sup>-1</sup>. This proportion of the eluents was kept stable for 7 min. Then the column was washed for 6 min with acetonitrile/water 95/5 v v<sup>-1</sup>. Afterwards the system was reequilibrated by washing the columns 4 min with 0.25 ml min<sup>-1</sup> acetonitrile/water 43/57 v v<sup>-1</sup>. SRL

**Table 1** Treatment scheme, body weight, bile salt-independent bile flow, and serum concentrations of bile salts, bilirubin, cholesterol, and triglycerides of the different groups. Means  $\pm$  s.e.mean of 10 animals each

Groups	1 controls	2	3	4	5	6
Daily oral doses over 14 days of	0.9% NaCl (1 ml kg <sup>-1</sup> ) p.o.	sirolimus (1 ml kg <sup>-1</sup> ) p.o.	0.9% NaCl (1 mg kg <sup>-1</sup> ) p.o.	0.9% NaCl (1 ml kg <sup>-1</sup> ) p.o.	sirolimus (1 mg kg <sup>-1</sup> ) p.o.	sirolimus (1 mg kg <sup>-1</sup> ) p.o.
Daily intraperitoneal doses over 14 days of	0.9% NaCl (6 ml kg <sup>-1</sup> ) i.p.	0.9% NaCl (6 ml kg <sup>-1</sup> ) i.p.	cyclosporine (10 mg kg <sup>-1</sup> ) i.p.	tacrolimus (1 mg kg <sup>-1</sup> ) i.p.	cyclosporine (10 mg kg <sup>-1</sup> ) i.p.	tacrolimus (1 mg kg <sup>-1</sup> ) i.p.
Body weight of the rats [g]						
before the first dose:	358.5 $\pm$ 9.5	353.7 $\pm$ 12.2	357.7 $\pm$ 6.4	315.3 $\pm$ 6.3*	384.5 $\pm$ 4.1*	321.1 $\pm$ 5.9*
after 1 week:	357.8 $\pm$ 9.2	354.1 $\pm$ 11.1	338.8 $\pm$ 6.7	291.3 $\pm$ 6.4*	347.4 $\pm$ 5.6	287.9 $\pm$ 5.6*
after 2 weeks:	364.1 $\pm$ 8.7	353.1 $\pm$ 11.2	323.3 $\pm$ 6.7*	287.9 $\pm$ 6.6*	290.8 $\pm$ 4.2*	262.9 $\pm$ 8.4*
Bile salt-independent bile flow [ $\mu$ l g l.w. <sup>-1</sup> min <sup>-1</sup> ]						
after 2 weeks:	0.67 $\pm$ 0.12	0.57 $\pm$ 0.13	0.36 $\pm$ 0.05*	0.43 $\pm$ 0.19	0.65 $\pm$ 0.06	0.64 $\pm$ 0.08
Bile acid serum concentration [ $\mu$ mol l <sup>-1</sup> ]						
before the first dose:	64.4 $\pm$ 12.1	71.0 $\pm$ 9.5	95.6 $\pm$ 4.4	56.2 $\pm$ 7.3	32.8 $\pm$ 3.8*	64.6 $\pm$ 6.7
after 1 week:	92.6 $\pm$ 8.4	65.8 $\pm$ 7.8*	205.7 $\pm$ 4.4*	58.3 $\pm$ 21.1*	79.4 $\pm$ 5.2	43.4 $\pm$ 5.1*
after 2 weeks:	109.6 $\pm$ 14.2	89.2 $\pm$ 7.8	317.6 $\pm$ 17.1*	109.8 $\pm$ 21.1	107.7 $\pm$ 6.5	53.6 $\pm$ 14.3*
Bilirubin serum concentration [ $\mu$ mol l <sup>-1</sup> ]						
before the first dose:	4.74 $\pm$ 0.38	2.98 $\pm$ 0.57*	4.68 $\pm$ 1.04	3.87 $\pm$ 0.74	3.47 $\pm$ 0.62	4.84 $\pm$ 1.00
after 1 week:	5.47 $\pm$ 0.81	4.95 $\pm$ 0.72	14.35 $\pm$ 2.08*	4.06 $\pm$ 0.76	21.00 $\pm$ 3.53*	5.81 $\pm$ 1.09*
after 2 weeks:	2.80 $\pm$ 0.48	3.93 $\pm$ 0.44	16.23 $\pm$ 2.20*	2.58 $\pm$ 0.41	16.83 $\pm$ 0.88*	4.19 $\pm$ 0.53*
Cholesterol serum concentration [mmol l <sup>-1</sup> ]						
before the first dose:	3.11 $\pm$ 0.09	2.69 $\pm$ 0.13*	3.15 $\pm$ 0.07	2.67 $\pm$ 0.11*	2.81 $\pm$ 0.09*	2.98 $\pm$ 0.11
after 1 week:	3.00 $\pm$ 0.09	3.61 $\pm$ 0.15*	3.37 $\pm$ 0.12*	3.12 $\pm$ 0.08	4.71 $\pm$ 0.23*	4.98 $\pm$ 0.29*
after 2 weeks:	2.89 $\pm$ 0.09	4.23 $\pm$ 0.15*	3.48 $\pm$ 0.08*	3.40 $\pm$ 0.05*	3.87 $\pm$ 0.16*	4.30 $\pm$ 0.28*
Triglyceride serum concentration [mmol l <sup>-1</sup> ]						
before the first dose:	0.29 $\pm$ 0.01	0.46 $\pm$ 0.03*	0.37 $\pm$ 0.03*	0.32 $\pm$ 0.02	0.57 $\pm$ 0.05*	0.42 $\pm$ 0.02*
after 1 week:	0.30 $\pm$ 0.02	0.75 $\pm$ 0.05*	0.42 $\pm$ 0.02*	0.45 $\pm$ 0.03*	0.92 $\pm$ 0.17*	0.82 $\pm$ 0.07*
after 2 weeks:	0.30 $\pm$ 0.01	0.67 $\pm$ 0.05*	0.40 $\pm$ 0.02*	0.55 $\pm$ 0.06*	0.76 $\pm$ 0.05*	0.55 $\pm$ 0.05*

\*Significant difference ( $P < 0.05$ ) compared to the controls. (l.w., liver weight).

**Table 2** Systematics of the Hawk's Cay Nomenclature (Consensus Document, 1999). The name of a metabolite consists of an 'A' for cyclosporine A, an 'M' to indicate that is is a metabolite and a sequence of arabic numbers (x) and letters indicating position and type of the amino acid of the cyclosporine molecule modified by metabolism.

Nomenclature	Modification
x	Hydroxylation of the indicated amino acid
xA1	Oxidation of the indicated amino acid to aldehyde
xA	Oxidation of the indicated amino acid to a carboxylic acid
xc	Hydroxylation and cyclization of the indicated amino acid
xN	N-Demethylation of the indicated amino acid
xOX	Formation of an epoxide at the indicated amino acid

and TRL were measured by liquid chromatography coupled with electrospray ionization/mass spectrometry (LC-ESI/MS) as previously described (Kirchner *et al.*, 2001) with slight modifications. EDTA whole blood samples (300  $\mu$ l) were treated with 600  $\mu$ l methanol/0.2 M ZnSO<sub>4</sub> (80/20 v v<sup>-1</sup>) plus 6  $\mu$ l 32-desmethoxyrapamycin (1 ng  $\mu$ l<sup>-1</sup>). Then samples were vortexed (30 s) and centrifuged (20000  $\times$  g, 5 min). Fifty  $\mu$ l of the supernatant was injected into the LC-ESI/MS system. A liquid chromatograph (LC) coupled with an electrospray ionization MS (LC/MSD 1100 SL Series, Agilent, Wald-

bronn, Germany) was used for all measurements. After injection the sample was transported by a stream of water (0.35 ml min<sup>-1</sup> for 3 min) to an extraction column (20  $\times$  2.1 mm, Waters Oasis<sup>®</sup> HLB, Eschborn, Germany). After turning the column-switching valve SRL, TRL, or CyA were eluted (flow: 0.25 ml min<sup>-1</sup>) from the extraction column in a back flash mode onto an analytical column (Hypersil C18, 5 $\mu$ , 250  $\times$  2 mm, phenomenex<sup>®</sup>, Aschaffenburg, Germany) by methanol/water (90/10 v v<sup>-1</sup>). After 20 min the extraction column was washed for 2 min with methanol and for 3 min with water before starting the next run. The temperature of the extraction and analytical columns was kept at 33°C. For single ion detection the mass spectrometer was focused on the [M + Na]<sup>+</sup> of SRL (m/z 936.6), TRL (m/z 824.0), CyA (m/z 1224.6) and 32-desmethoxyrapamycin (m/z 906.6) in the positive ion mode. The accuracy of the sirolimus, tacrolimus, and cyclosporine measurement was controlled by monthly participation in the International Proficiency Testing Scheme (Analytical Services International, London, U.K.).

**Statistics** Results are expressed as means  $\pm$  s.e.mean. The data were compared by analysis of variance. When the analysis indicated a significant difference between the controls and the experimental group, the means were compared using Dunnett's multiple comparisons test. A probability of  $< 0.05$  was defined as significant.

## Results

### *Effects of SRL, CyA, TRL, SRL/CyA, and SRL/TRL on the body weight of the rats*

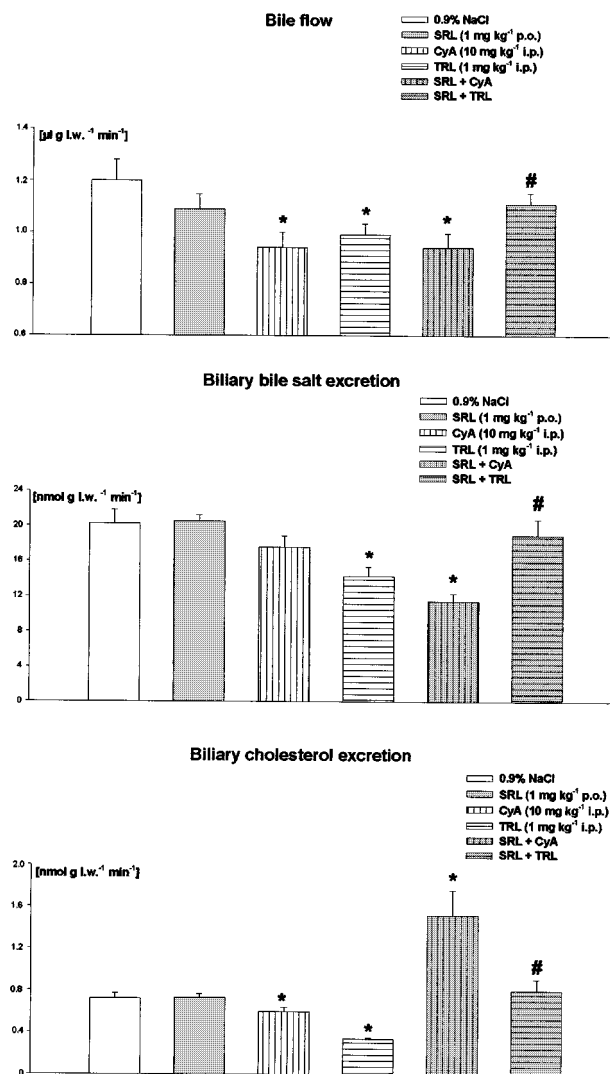
During the daily treatment of the rats with immunosuppressants over 2 weeks none of the animals died. However, concerning the loss of body weight, big differences between the single animal groups could be observed. While in the controls and the SRL-treated rats the body weight of the rats was stable between 353 and 364 g, the body weight of the CyA- and TRL-treated rats was reduced by 9 and 10% within 2 weeks (Table 1). The loss of body weight was amplified after combining SRL with either CyA (−25%) or TRL (−18%).

### *Effects of SRL, CyA, TRL, SRL/CyA, and SRL/TRL on bile flow and biliary excretion of bile salts and cholesterol*

The bile flow was  $1.20 \pm 0.08 \mu\text{l}$  per gram liver weight per min ( $\mu\text{l g l.w.}^{-1} \text{ min}^{-1}$ ) in the controls and was reduced by SRL (−10%; statistically not significant), CyA (−22%), as well as by TRL (−18%) (Figure 1). When SRL was combined with CyA the bile flow was decreased to the same degree as by CyA given alone. After combination of SRL with TRL the bile flow was only slightly attenuated by 7.5% compared to the control values (Figure 1). The biliary excretion of bile salts ( $20.3 \pm 1.6 \text{ nmol g l.w.}^{-1} \text{ min}^{-1}$  in the controls) was diminished by CyA (−15%) as well as by TRL (−36%), but not affected by SRL (Figure 1). Cotreatment of SRL with CyA led to a decrease of biliary bile salt excretion by 44% of the controls. However, this parameter was not altered by the combination of SRL with TRL (Figure 1). The biliary excretion of cholesterol was  $0.72 \pm 0.05 \text{ nmol g l.w.}^{-1} \text{ min}^{-1}$  and was attenuated by CyA (−16%) as well as by TRL (−47%), but not influenced by SRL (Figure 1). Surprisingly, we observed a 2 fold increase of the biliary cholesterol excretion in the SRL/CyA-treated group. On the other hand, this parameter remained unchanged compared to the controls after simultaneous administration of SRL and TRL (Figure 1).

### *Effects of SRL, CyA, TRL, SRL/CyA, and SRL/TRL on serum concentrations of bile salts, bilirubin, cholesterol, and triglycerides*

In the controls the serum concentration of bile salts was determined as  $109.6 \pm 14.2 \mu\text{mol l}^{-1}$ . We observed a 3 fold increase of serum bile salt concentration only in those animals treated with CyA (Table 1). When rats were given either SRL, TRL, or SRL/CyA, bile salt serum concentration remained constant and after the cotreatment of SRL with TRL this parameter was even reduced to the half (Table 1). Bilirubin serum concentration ( $2.80 \pm 0.48 \mu\text{mol l}^{-1}$ ) was 5 fold increased by CyA. Neither SRL nor TRL led to a significant change of this parameter. Cotreatment with SRL and CyA enhanced the bilirubin serum concentration to the same degree as CyA alone. After the combination of SRL with TRL a less pronounced increase (+49%) of bilirubin serum concentration was measured (Table 1). Serum cholesterol concentration ( $2.89 \pm 0.09 \text{ mmol l}^{-1}$ ) was elevated after treatment with SRL (+46%), CyA (+18%) as well as with TRL (+18%). The SRL induced increase of cholesterol

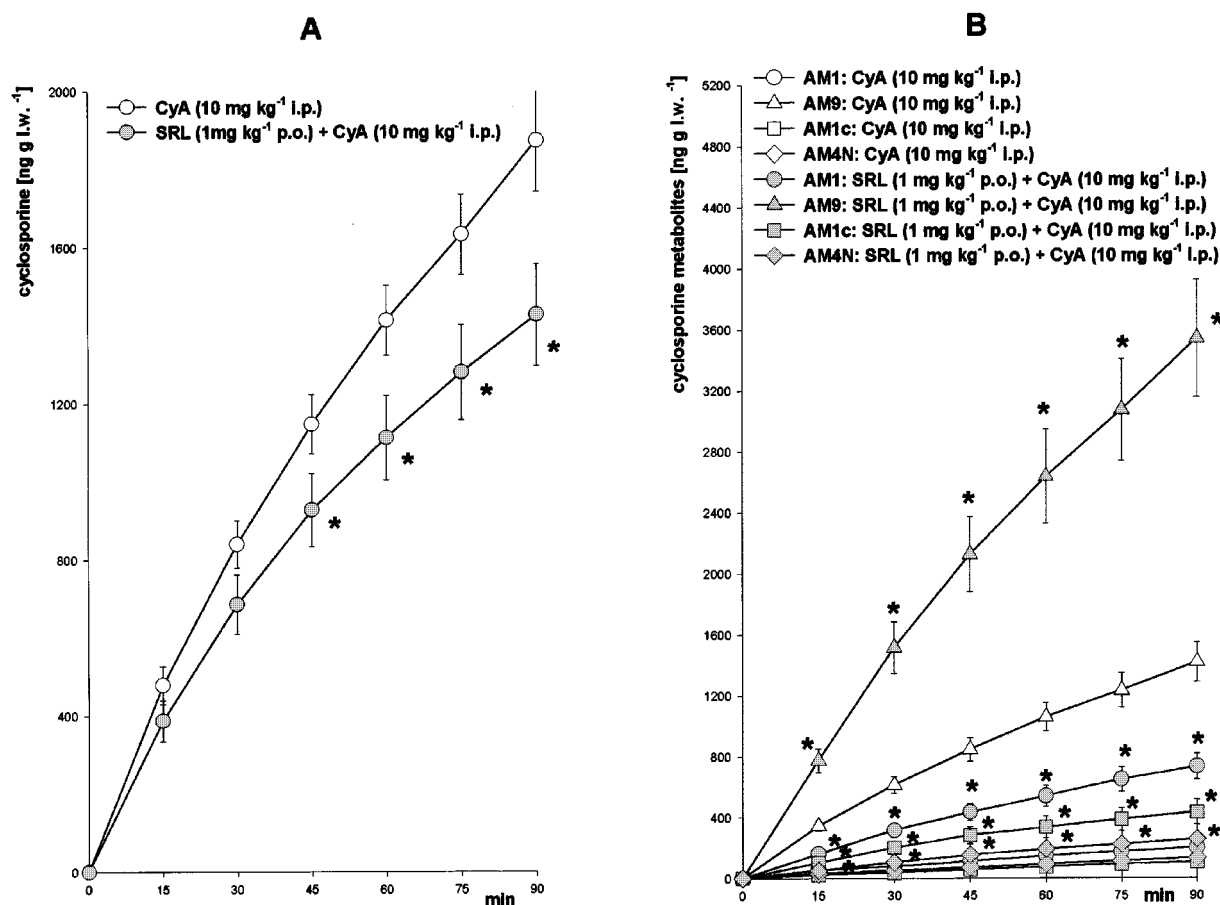


**Figure 1** Bile flow and biliary excretion of bile salts and cholesterol after daily treatment over 2 weeks with sirolimus (SRL,  $1 \text{ mg kg}^{-1} \text{ p.o.}$ ), cyclosporine (CyA,  $10 \text{ mg kg}^{-1} \text{ i.p.}$ ), tacrolimus (TRL,  $1 \text{ mg kg}^{-1} \text{ i.p.}$ ) and of SRL ( $1 \text{ mg kg}^{-1} \text{ p.o.}$ ) plus CyA ( $10 \text{ mg kg}^{-1} \text{ i.p.}$ ) or TRL ( $1 \text{ mg kg}^{-1} \text{ i.p.}$ ). Means  $\pm$  s.e. mean of 10 animals each. \*Significant difference ( $P < 0.05$ ) compared to the controls. #Significant difference ( $P < 0.05$ ) between SRL + CyA- and SRL + TRL-treated groups. (l.w., liver weight).

serum concentration was neither exacerbated by cotreatment with CyA nor with TRL (Table 1). A similar picture was seen after determination of triglyceride serum concentration. Triglyceride serum concentration ( $0.30 \pm 0.01 \text{ mmol l}^{-1}$ ) was again enhanced by SRL (+123%), CyA (+33%) as well as by TRL (+83%). When SRL was combined with CyA triglyceride serum concentration was 13% higher than in the SRL-treated animals and 17% lower after combination with TRL (Table 1).

### *Interactions of SRL/CyA and SRL/TRL on their blood concentration and biliary excretion*

Biliary excretion and blood concentrations of the immunosuppressive drugs used were analysed by sensitive and specific chromatographic procedures. In the animal groups treated



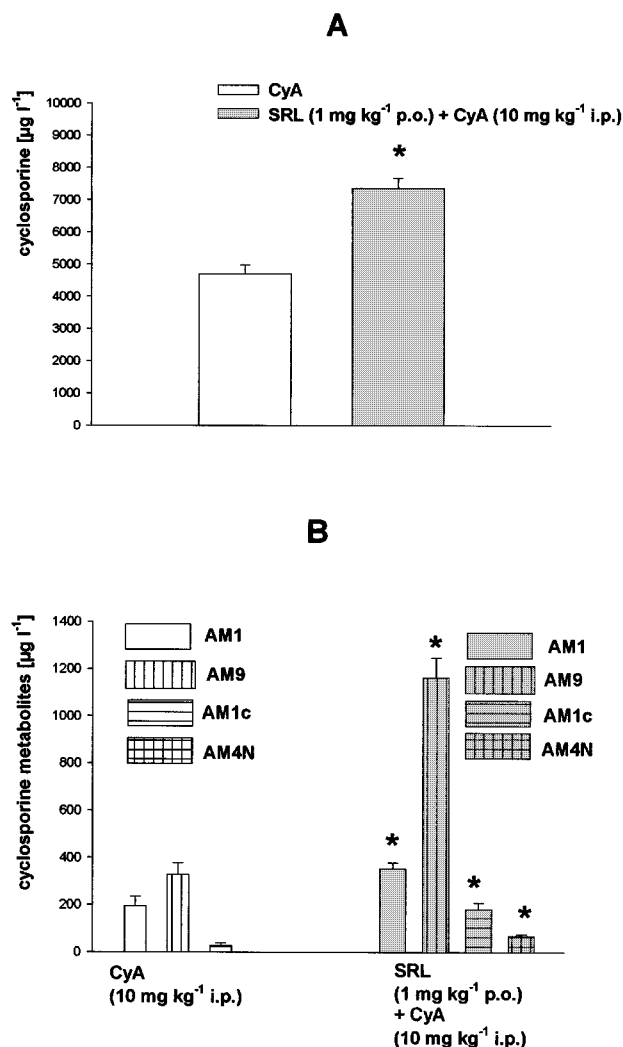
**Figure 2** Biliary excretion of (A) cyclosporine and (B) its metabolites after daily treatment over 2 weeks with cyclosporine (CyA, 10 mg kg<sup>-1</sup> i.p.) or with SRL (1 mg kg<sup>-1</sup> p.o.) plus CyA (10 mg kg<sup>-1</sup> i.p.). Means  $\pm$  s.e.mean of 10 animals each. \*Significant difference ( $P < 0.05$ ) compared to the CyA-treated group. (l.w., liver weight).

with a single immunosuppressant the mean blood level of SRL was  $2.8 \pm 0.3 \mu\text{g l}^{-1}$  (Figure 2A), of CyA  $4703 \pm 285 \mu\text{g l}^{-1}$  (Figure 3A), and of TRL  $7.1 \pm 0.6 \mu\text{g l}^{-1}$  (Figure 4B), respectively. When SRL was given simultaneously with CyA or TRL, the mean blood level of SRL was significantly increased 5 fold by CyA but not changed by TRL (Figure 4A). The biliary excretion of SRL ( $2.2 \pm 0.3 \text{ pg g l.w.}^{-1} \text{ min}^{-1}$ ) was enhanced 25 fold by CyA but was not affected by TRL (Figure 4C). On the other hand, SRL increased blood concentration of CyA by 56% (Figure 3A) and of TRL by 33% (Figure 4B). The blood concentrations of the main CyA metabolites AM1 ( $195 \pm 41 \mu\text{g l}^{-1}$ ), AM9 ( $328 \pm 49 \mu\text{g l}^{-1}$ ), AM1c ( $26 \pm 11 \mu\text{g l}^{-1}$ ), and AM4N (not detectable in the controls) were elevated 2–5 fold (Figure 3B) by SRL, as well. Whereas the biliary excretion of CyA ( $22.1 \text{ ng g l.w.}^{-1} \text{ min}^{-1}$ ) was reduced by 20% (Figure 2A) and the excretion of the CyA metabolites was 2–4 fold increased by SRL (Figure 2B), the biliary excretion of TRL ( $2.7 \pm 0.8 \text{ pg g l.w.}^{-1} \text{ min}^{-1}$ ) was only slightly attenuated by SRL (Figure 4D).

## Discussion

Cholestatic properties of CyA have been observed in clinical studies (Kahan, 1989) and in experiments with rats (Le Thai

*et al.*, 1988). Inhibition of the transporters involved in bile formation is a documented mechanism for the cholestatic effects of CyA (Mosley & Morrisette, 1990). Hepatic uptake of conjugated bile salts depends on  $\text{Na}^+$  and is mediated by Ntcp ( $\text{Na}^+$ /taurocholate cotransporting polypeptide). The transport of unconjugated bile salts and other organic anions into hepatocytes is mediated by a transporter family called Oatps (organic anion transporting polypeptides). The biliary excretion of both conjugated and unconjugated bile salts depends on ATP. The excretion of taurine-conjugated bile salts is mediated by Bsep (bile salt export pump) and that of divalent glucuronated or sulphated bile salts by Mrp2 (multidrug resistance protein2) (Müller & Jansen, 1998). CyA was shown to decrease both the uptake of bile salts in the liver (Stacey & Kotecka, 1988) and their excretion into the bile canaliculi. The inhibitory effects of CyA on bile salt transporters of the canalicular hepatocyte membrane are described as being stronger than those on transporters of the basolateral hepatocyte membrane (Böhme *et al.*, 1994). In experiments with vesicles isolated from Bsep-expressing Sf6 cells CyA inhibited Bsep-mediated transport of bile salts (Stieger *et al.*, 2000). Bile salt-independent bile flow is mainly driven by the biliary excretion of bicarbonate and reduced glutathione (GSH) which is substrate of Mrp2 (Paulusma *et al.*, 1999). In single and short-term treatment studies with rats it was observed that CyA-induced cholestasis was due not



**Figure 3** Blood concentrations of (A) cyclosporine and (B) its metabolites after daily treatment over 2 weeks with cyclosporine (CyA, 10 mg kg<sup>-1</sup> i.p.) or sirolimus (SRL, 1 mg kg<sup>-1</sup> p.o.) plus CyA (10 mg kg<sup>-1</sup> i.p.). Means  $\pm$  s.e. mean of 10 animals each. \*Significant difference ( $P < 0.05$ ) compared to the CyA-treated group.

only to alterations in the hepatobiliary transport of bile salts but also to an impairment of bile formation dependent on the biliary secretion of glutathione (Moran *et al.*, 1998). In our experiments daily treatment of the rats over 2 weeks with CyA led to a decrease of bile salt dependent ( $-15\%$ ) as well as of bile salt-independent bile flow ( $-46\%$ ) (Figure 1; Table 1). Bile salts play an important role in the biliary excretion of cholesterol (Graham *et al.*, 1989). Therefore, the observed decrease of biliary cholesterol excretion ( $-16\%$ ) by CyA in our and other studies with rats (Galan *et al.*, 1995) could be the result of impaired biliary bile salt excretion induced by CyA (Figure 1). As indirect parameters of cholestasis a 3 fold enhanced bile salt serum concentration and more than a 5 fold increase of bilirubin serum concentration compared to the controls were measured in the CyA-treated rats (Table 1).

Previously, contradictory results have been reported with respect to the effect of TRL on bile flow. In a study investigating the effects of chronic tacrolimus treatment (0.8 mg kg<sup>-1</sup>) on bile secretion in rats for 6 weeks, TRL was found to induce cholestasis by inhibiting primarily the

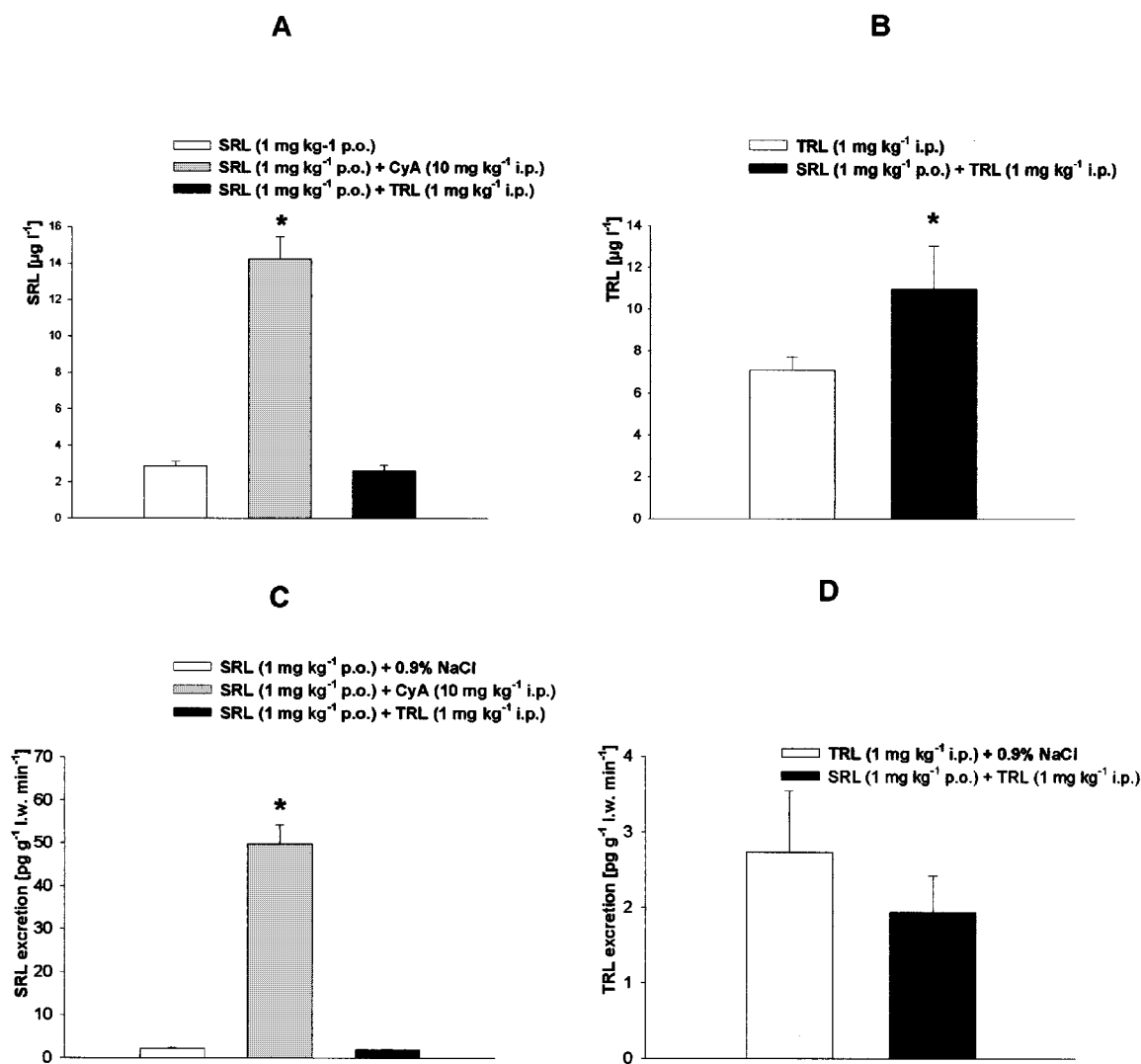
biliary excretion of GSH and, to lesser extent, of bicarbonate (Sanchez-Campos *et al.*, 1998). In another study, rats were given TRL up to 4 mg kg<sup>-1</sup> for 7 days. In this study TRL increased bile salt-dependent flow while bile salt-independent flow was reduced by TRL (Mizuta *et al.*, 1999). When rats were treated with either a high dose of 10 mg kg<sup>-1</sup> TRL only once or daily for 1 week TRL even induced choleresis through stimulation of insulin-like growth factor-I production in the liver of rats (Kawamura *et al.*, 2001). When we administered rats TRL at a dose of 1 mg kg<sup>-1</sup> i.p. bile flow ( $-18\%$ ) as well as biliary excretion of bile salts ( $-36\%$ ) and cholesterol ( $-47\%$ ) were reduced to a similar extent as in the experiments with CyA (Figure 1). TRL diminished the bile salt-independent bile flow statistically not significantly by 35% (Table 1). Surprisingly, in spite of impaired biliary excretion of bile salts no increase of serum bile salts concentration was seen in the TRL-treated animals (Table 1). From this observation we hypothesize that TRL may decrease bile salt synthesis *via* inhibition of the microsomal enzyme cholesterol-7 $\alpha$ -hydroxylase, the key enzyme in bile salt synthesis. However, no effect of TRL on this enzyme has been reported in the literature so far.

Information regarding the effects of SRL on bile flow is very limited at the moment. In preliminary own experiments with rats we could show that a single dose of 6 mg kg<sup>-1</sup> i.p. SRL resulted in a decrease of bile flow mainly due to an impaired excretion of GSH (Deters *et al.*, 2001). Coincident with these results SRL was shown to decrease mRNA expression of the transport protein Mrp2 in the liver of rats (Bramow *et al.*, 2001). In our present study the oral treatment of SRL at a dose of 1 mg kg<sup>-1</sup> day<sup>-1</sup> attenuated bile flow by 10%. This effect can be contributed mainly to the statistically not significant reduction of bile salt-independent bile flow ( $-15\%$ ; Table 1) because SRL neither impaired biliary excretion of bile salts nor of cholesterol (Figure 1). Compatible to these results, the serum bile salt concentration was not and the serum bilirubin concentration was only slightly increased in the SRL-treated rats (Table 1).

When SRL was combined with CyA the bile flow was reduced to the same extent as in the experiments where CyA was given alone (Figure 1). The CyA-induced decrease of biliary bile salt excretion was exacerbated by cotreatment with SRL. On the other hand, the decrease of biliary bile salt excretion after SRL/CyA cotreatment did not result in elevated serum concentrations of bile salts as observed after CyA-treatment indicating reduced synthesis of bile salts out of cholesterol. Results from earlier studies indicate that CyA may alter the binding kinetics of low-density lipoproteins (LDL) to the LDL-receptor, thereby interfering with the normal feedback mechanisms that regulate cholesterol biosynthesis (Hoogeveen *et al.*, 2001). Both mechanisms, decrease of cholesterol metabolism and increase of cholesterol synthesis, could cause a strong increase of the hepatic cholesterol concentration and consequently of the biliary cholesterol excretion as observed in our experiments (Figure 1).

These effects were not observed in the SRL- or CyA-treated rats, because in these animals the SRL or CyA blood concentrations were much lower than in the SRL/CyA-treated rats (Figures 3 and 4).

When SRL was combined with TRL bile flow was slightly attenuated and the biliary excretion of bile salts and



**Figure 4** Blood concentrations and biliary excretion of (A,C) sirolimus and (B,D) tacrolimus after daily treatment over 2 weeks with sirolimus (SRL,  $1 \text{ mg kg}^{-1} \text{ p.o.}$ ), tacrolimus (TRL  $1 \text{ mg kg}^{-1} \text{ i.p.}$ ) and of SRL ( $1 \text{ mg kg}^{-1} \text{ p.o.}$ ) plus CyA ( $10 \text{ mg kg}^{-1} \text{ i.p.}$ ) or TRL ( $1 \text{ mg kg}^{-1} \text{ i.p.}$ ). Means  $\pm$  s.e.mean of 10 animals each. \*Significant difference ( $P < 0.05$ ) compared to the SRL- or TRL-treated group. (l.w., liver weight).

cholesterol was not impaired in comparison to the controls (Figure 1). Bile salt serum concentration in the SRL/TRL group decreased to the half of the control values while the bilirubin serum concentration was slightly elevated (Table 1).

The first clinical studies comparing the effects of SRL/TRL and SRL/CyA on lipid serum concentration indicate lower serum concentrations of both cholesterol and triglycerides after SRL/TRL than after SRL/CyA treatment in transplant recipients (McAlister *et al.*, 2000; Trotter *et al.*, 2001). However, it has been reported that TRL also caused elevated cholesterol and triglyceride serum concentrations (Ichimaru *et al.*, 2001; Charco *et al.*, 1999). In our experiments cholesterol serum concentration was elevated by SRL, CyA, TRL, as well as by SRL/CyA and SRL/TRL. The highest cholesterol serum concentration was observed after treatment of SRL followed by CyA and TRL which showed the same effect on cholesterol serum concentration. Neither cotreatment of SRL with CyA nor with TRL further increased SRL-induced hypercholesterolaemia (Table 1). Probably, in the

case of SRL/CyA cotreatment, a further increase of the SRL-induced hypercholesterolaemia after addition of CyA could be partially compensated by the strong increase of the biliary cholesterol excretion (Figure 1).

The effects of the immunosuppressants on serum triglyceride concentrations were similar to those on cholesterol concentrations. In our rat model all immunosuppressants induced hypertriglyceridaemia. The highest triglyceride serum concentration was measured after SRL treatment followed by TRL and CyA (Table 1). After coadministration of SRL with CyA the triglyceride serum concentration was a little bit higher than in the rats treated with SRL alone. On the other hand, in SRL/TRL-treated animals a somewhat lower triglyceride serum concentration than after SRL or SRL/CyA treatment was determined (Table 1). CyA was described to reduce LDL-receptor activity in a hepatocellular tumour line (al Rayes *et al.*, 1997). In experiments with normolipidaemic New Zealand White rabbits tacrolimus decreased the clearance of radiolabelled human LDL and very low-density

lipoproteins (VLDL) being injected into the animals (Baier *et al.*, 2000). A similar mechanism has been postulated for the hyperlipidaemic effects of sirolimus. In clinical studies in renal transplant patients SRL could induce both hypercholesterolemia and hypertriglyceridaemia. The authors concluded from their results that these hyperlipidaemic effects of SRL were not caused by an enhanced hepatic VLDL production, but rather by a decrease in the clearance of ApoB100-containing lipoproteins (Hoogveen *et al.*, 2001). These differences in the mechanisms of immunosuppressant-induced hypertriglyceridaemia (reduction of LDL-receptor activity in the case of CyA-induced and reduction of LDL clearance in the case of SRL- and TRL-induced hypertriglyceridaemia) may explain the slightly elevated triglycerid serum concentrations in the SRL/CyA-treated animals in comparison to the SRL- and SRL/TRL-treated rats (Table 1). Inducing effects of SRL on key enzymes of the synthesis of fatty acid and cholesterol could serve as further explanation of the observed hyperlipidaemic effects of SRL, but have not been proven in *in vitro* experiments, so far.

In our experiments SRL increased blood concentrations of CyA (Figure 3A) as well as of CyA metabolites (Figure 3B). This finding cannot solely be explained by the inhibition of CyA metabolism by SRL according to CYP3A4 pathway, because in this case one should observe elevated CyA blood concentrations with simultaneously decreased blood concentrations of its metabolites. An explanation for this phenomenon could be that the biliary excretion of CyA was decreased by 20% by SRL (Figure 2A) implicating the involvement of a transport system for CyA into the bile (for example P-glycoprotein). Additionally, the reabsorption of CyA in the gut may also be increased in the presence of SRL by inhibition of P-glycoprotein or CYP3A4 in the mucosa cells (Lampen *et al.*, 1998). Both mechanisms would lead to higher amounts of CyA in the body, finally resulting in higher blood concentrations of CyA metabolites, too. Interactions on biliary excretion of SRL with other drugs that are eliminated mainly *via* bile such as statines, protease inhibitors, or some sulfonylurea antidiabetica could be of importance, as well, in clinical situations. The biliary excretion of the CyA metabolites, however, was not impaired by SRL (Figure

2B) indicating that their transport into the bile is not specific or, alternatively, mediated by a transporter not involved in CyA elimination.

The TRL blood concentration was increased by SRL, as well (Figure 4B). Because the biliary excretion of TRL was only slightly reduced by SRL (Figure 4D), we conclude that inhibition of TRL metabolism plays a more important role for the elevated TRL blood concentrations than its decreased biliary elimination. CyA increased SRL blood concentration as well as biliary excretion of SRL, which can be explained by reduction of SRL metabolism by CyA. On the other side, neither SRL blood concentrations nor biliary SRL excretion were affected by TRL although both substances bind with the same  $K_s$  values ( $1-2 \mu\text{mol l}^{-1}$ ) to purified reconstituted human CYP3A4 with similar velocities of main metabolites formation (Sattler *et al.*, 1992).

In summary, we showed in the subchronic bile fistula model in the rat that SRL, CyA, as well as TRL have cholestatic properties and were able to induce both hypercholesterolemia and hypertriglyceridaemia after daily treatment with these immunosuppressants over 2 weeks. SRL/CyA increased serum cholesterol concentrations to the same extent as SRL/TRL did. Serum triglyceride concentrations were a little bit more increased by SRL/CyA than SRL/TRL, whereas bile flow was significantly less decreased by SRL/TRL than by SRL/CyA. SRL led to an increase of both CyA and TRL blood concentrations. However, SRL blood concentration was only increased by CyA but not by TRL. Therefore, SRL/TRL could be the better pharmacological regimen than SRL/CyA in cholestatic patients and in those facing difficulties to reach the narrow therapeutic range of SRL.

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