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Hepatocellular Effects of Cyclosporine A and its Derivative SDZ IMM 125 *in Vitro*

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ABSTRACT

The novel immunosuppressive drug O-hydroxyethyl-D(Ser)^8-cyclosporine (SDZ IMM 125) and cyclosporine A (CyA) were compared in different *in vitro* models with respect to hepatocellular side effects. SDZ IMM 125 was less lipophilic than CyA and also decreased liposomal membrane anisotropy less. Furthermore, SDZ IMM 125 increased Na^+ and Ca^{++} permeability across the liposomal membranes significantly more than CyA. The uptake of CyA and SDZ IMM 125 into freshly isolated rat hepatocytes was neither saturable, Na^+ dependent or temperature sensitive, nor could it be inhibited vice versa, indicating passive diffusion. The diffusion coefficient of CyA was about two times higher than that of SDZ IMM 125, reflecting its higher lipophilicity. In primary hepatocyte monolayers the cellular concentrations of CyA were about two times higher than that of SDZ IMM 125. As an indicator of cholestasis the saturable uptake of cholyltaurine into isolated cells was found to be apparently competitively inhibited to the same extent by both compounds. In isolated perfused rat livers SDZ IMM 125 caused a significantly greater decrease in bile flow than did CyA. Release of lactate dehydrogenase from hepatocyte primary cultures and from isolated perfused livers were determined as parameter of cell damage. In both systems the cytotoxicity of SDZ IMM 125 was significantly higher than that of CyA. The data suggest that SDZ IMM 125 causes greater cholestatic and cytotoxic effects than CyA at equimolar cellular exposure.

The undecapeptide CyA (Sandimmun) has found a particular clinical interest because of its immunosuppressive properties. It provides beneficial treatment of autoimmune diseases and prevents allograft rejection in organ transplantation (Borel *et al.*, 1976; Cohen *et al.*, 1984; Rogers and Kahan, 1984). The immunosuppressive medication, however, requires careful monitoring by reason of therapy associated side effects of CyA. In addition to the nephrotoxicity (Kahan, 1989; Mason, 1990), increased bilirubin plasma levels and elevated serum bile acid levels have been reported from CyA-treated patients (Schade *et al.*, 1983; Rodger *et al.*, 1983; Atkinson *et al.*, 1983) and animal experiments (Roman *et al.*, 1990, Stone *et al.*, 1988), indicating cholestatis as a side effect of the drug. The interaction of CyA with the hepatic transport of bile acids is discussed controversially. Both competitive (Moseley *et al.*, 1990) and noncompetitive (Zimmerli *et al.*, 1987) inhibition of cholyltaurine uptake by CyA have been observed. Studies with isolated rat hepatocytes (Ziegler and Frimmer, 1986) suggested a nonspecific binding of CyA to protein components of a postulated cholate transport system without uptake by this system. In rats, at CyA dose-levels of 80 mg/kg, serum transaminase activities were slightly increased, which seemed to be linked to unspecific toxic effects of the drug. Increased serum activities of ALT and AST were not observed in healthy volunteers nor in transplant patients (Mason, 1990; Schade *et al.*, 1983; Ellis *et al.*, 1986; Farthing *et al.*, 1981).

The novel derivative SDZ IMM 125 is almost equipotent to CyA concerning its immunosuppressive properties, but it caused less renal dysfunction than CyA (Donatsch *et al.*, 1992; Hiestand *et al.*, 1993). The drug was also well tolerated in healthy volunteers and in psoriatic patients at doses of less than 400 mg. At higher dose-levels SDZ IMM 125 increased the serum activities of ALT and AST (Witkamp *et al.*, 1995).

In our study we compared CyA and SDZ IMM 125 in different hepatic *in vitro* models to determine the cytotoxic and cholestatic potential of both drugs. Because their physicochemical properties might have an impact on their hepatocellular effects, both compounds were also studied in liposomal membranes in addition to experiments with isolated rat hepatocytes, hepatocyte primary cultures and isolated perfused livers.

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ABBREVIATIONS: CyA, cyclosporine A; SDZ IMM 125, O-hydroxyethyl-D(Ser)^8-cyclosporine; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DPH, 1,6-diphenyl-1,3,5-hexatriene; DOPC, dioleyl-phosphatidylcholine.

Materials and Methods

Chemicals. CyA, SDZ IMM 125, ^{3}H-labeled CyA with a specific activity of 9.3 Ci/mmol, and ^{14}C-labeled SDZ IMM 125 with a specific activity of 51.7 mCi/mmol were obtained from the Preclinical Research Department, Novartis Pharma AG, Basle, Switzerland. [6-^{3}H]-cholyltaurine (Na^{+}-salt) with a specific activity of 6.6 Ci/mmol and [2,4-^{3}H]-cholic acid with a specific activity of 12 Ci/mmol were obtained from Du Pont-New England Nuclear (Dreieich, Germany). ^{22}NaCl with a specific activity of 500 mCi/mg Na^{+} and $^{45}CaCl_2$ with a specific activity of 25 mCi/mg Ca^{++} were from Amersham (Buckinghamshire, UK). Collagenase "Worthington" CLS II with a specific activity of 125–150 U/mg protein was obtained from Biochrom KG (Berlin, Germany). 1,6-Diphenyl-1,3,5-hexatriene was from Molecular Probes, Inc. (Eugene, OR). All other substances were obtained at the highest purity from commercial sources.

Liposome preparation. Liposomes were prepared by the extrusion technique, as described previously (MacDonald *et al.*, 1991). dioleyl-phosphatidylcholine was used as phospholipid. The distribution of liposome size was determined by laser light scattering at 90°C with a ZetaSizer III (Malvern Instruments, London, UK).

Partition coefficient measurements. Appropriate amounts of CyA or SDZ IMM 125 were dried from stock solutions in chloroform into eppendorf vials. One milliliter of liposome suspension (total lipid = 10 mg) was added to the vial and shaken for 2 hr. Concentrations of CyA or SDZ IMM 125 in the liposomal solution were between 12 and 320 μM. Centrifugation tubes were equilibrated over night with a corresponding aqueous concentration to avoid excessive adsorption of CyA to the tube. The suspension was centrifuged in a Beckman Ultracentrifuge TL-100 at 198.000 rpm for 6 hr at 25°C. The lipid content was measured by a phospholipase-D/cholinoxidase/PAP-test (WAKO Chemicals GmbH, Neuss, Germany). CyA concentration in supernatant and pellet was measured by a radioimmunoassay (detection limit: 15 ng/ml).

Fluorescence measurements. Steady-state Fluorescence measurement were performed with a Perkin-Elmer LS50B instrument (Perkin Elmer Inc., Basle, Switzerland) equipped with polarisation accessories. The cuvette holder was thermostated by a Julabo F10 thermostat (Merck ABS, Switzerland) at 25°C. Excitation was done at 336 nm (3 nm slit width), emission was determined over a 10-sec integration at 430 nm (3-nm slit width). DPH was added to the liposomal suspension to give a molar lipid: DPH ratio of 200 in a buffer system of 50 mM NaCl, 50 mM KCl, 20 mM Tris, pH 7.4. The LS-50B software allowed the calculation of anisotropy with G-factor correction.

Ion flux measurements. Liposomes were suspended in 60 μl buffer containing 150 mM NaCl, 30 mM imidazole, 5 mM $MgSO_4$, 1 mM EDTA, pH 7,2 and 2 μCi ^{22}NaCl or $^{45}CaCl_2$, respectively. The suspensions were preequilibrated with the test compound (CyA or SDZ IMM-125). For that reason, 1 to 4 μl of the test compound stock solution (10 mM in ethanol:H_2O) were added to 60 μl liposomes in different test vials. As control experiment, the same amount of ethanol:H_2O without test compound was added to 60 μl of the liposome suspension. The flux experiments were performed at 20 to 23°C. An ion exchange resin (Bio-Rex 70) was equilibrated for at least one day with 1 mM Tris, pH 7.4. For at least 2 days the resin was reequilibrated with 0.15 M Tris, pH 7.4. After filling the microcolumns with the resin, the columns were washed with 2 ml of the appropriate iso-osmolar sucrose solution, containing 342 mM sucrose, 20 mM Tris, pH 7.2 for Na^{+}-flux measurement. The prepared microcolumns could be stored at 4°C for several days.

At time 0, the liposomes were added to the radioactive test compound, thoroughly mixed and kept at room temperature or at 37°C. At different time points (0–200 hr) 6 μl of the liposome suspension was added to 60 μl of cold (0–2°C) iso-osmolar sucrose and mixed well. From that mixture, 28-μl aliquots were layered on the cooled microcolumns. The liposomes from the sample were directly eluted with 1 ml cold (0–4°C) iso-osmolar sucrose into a scintillation counting vial. A total of 4 ml of scintillation fluid was added and the radioactivity was measured for 60 min or until the relative S.D. was less than 0.4%. As control experiment, 5 ml of the liposome-sucrose mixture were mixed with 1 ml of sucrose and the radioactivity was determined.

Animals. All animal experiments were approved by the Cantonal authorities following the guidelines of the Swiss Animal Welfare Act. Male Wistar rats, weighing approximately 300 g (BRL, Fühlinsdorf, CH) were used throughout all studies. The animals had free access to a standard diet and tap water and were kept in a constant temperature environment with natural day and night rhythm. They were anaesthetised by i.p. injection of urethane (1 g/kg) before hepatectomy.

Isolated perfused rat liver. Livers were isolated as described (Fricker *et al.*, 1994). They were perfused in a noncirculating system with Krebs-Henseleit medium saturated with carbogen (95% O_2/5% CO_2) and adjusted to pH 7.4 at a flow rate of 3 ml $\times$ min^{-1} $\times$ (g liver)$^{-1}$ and a hydrostatic pressure of 12 $\pm$ 1 cm water. The perfusion medium consisted of 1.25 mM $CaCl_2$, 118 mM NaCl, 4.74 mM KCl, 1.19 mM $MgCl_2$, 24.90 mM $NaHCO_3$, 0.59 mM Na_2HPO_4, 0.59 mM KH_2PO_4 and 5.50 mM D-glucose, pH 7.4. In addition, the perfusion medium was supplemented with 62 μM cholyltaurine to maintain a constant bile flow. All perfusion media contained a final concentration of 1% DMSO and 10% fetal calf serum, which was necessary to dissolve the highly lipophilic cyclosporins at the desired concentration. The common bile duct was cannulated by a Teflon tube prior to onset of the perfusion. Bile was collected during the perfusion period in 5-min intervals and the volume secreted over time was determined gravimetrically. After an initial recovery phase of 30 min, when the bile flow remained stable, the perfusion with medium containing either CyA or SDZ IMM-125 at concentrations of 1, 5, 10 and 50 μM was started.

Isolation of hepatocytes. Hepatocytes were prepared by a modified collagenase perfusion technique (Schramm *et al.*, 1993). The cells were suspended in supplemented Krebs-Henseleit medium. For uptake studies in Na^{+}-depleted medium, sedimented hepatocytes were washed three times with choline buffer containing 118 mM choline chloride, 4.74 mM KCl, 1.2 mM $MgCl_2$, 0.59 mM KH_2PO_4, 0.59 mM K_2PO_4, 24 mM choline hydrogen carbonate, 1.25 mM $CaCl_2$ and 5.5 mM D-glucose, which was saturated with carbogen and adjusted to pH 7.4. The cell concentration was 2 to 3 $\times$ 10^6 cells $\times$ ml^{-1}. Before uptake experiments the hepatocytes were allowed to recover for approximately 30 min by gently shaking them under a carbogen atmosphere at 37°C. The viability of the cells was estimated immediately after the isolation procedure and a second time after the transport experiments by determination of Trypan blue exclusion. Only cells with a viability >90% were used for transport experiments. All experiments with freshly isolated cells were performed within 2 hr after hepatocyte isolation.

Primary hepatocyte cultures. Freshly isolated hepatocytes were resuspended in William's medium (Williams and Gunn, 1974), which was supplemented with 10% fetal calf serum, 0.1 mM insulin, 0.1 mM dexamethason, 2 mM glutamine and 100 U/liter penicillin/streptomycin (Wolf *et al.*, 1997). The suspension was carefully sieved through filters with a mesh size of 520, 280, and 190 mm. Then, the cell suspension was placed on ice for 15 min to allow the viable parenchymal cells to sediment. The supernatant was removed and replaced by fresh medium. This procedure was repeated twice, then the viability of the cells was determined by Trypan-blue exclusion. Only cell suspensions with more than 90% of the cells excluding the dye were used. The cells were placed in 60-mm culture dishes (Primaria Becton-Dickinson, Oxford, CA) at a cell density of 2 $\times$ 10^6 cells/3 ml at 37.5°C and under carbogen (95% air, 5% CO_2) atmosphere. After 3 hr, the medium was removed and replaced by fresh medium. Test compound was given together with new medium. Therefore, the cyclosporins were dissolved in DMSO and this solution was added to the culture medium resulting in a final concentration of 1% DMSO in the culture conditions. Control plates received the DMSO containing medium without cyclosporine.

Transport experiments. Uptake of cyclosporines and cholyltaurine into freshly isolated hepatocytes was measured at 37°C by a rapid centrifugation technique (Anwer *et al.*, 1966; Schramm *et al.*, 1993). Uptake measurements were started by the addition of 600 μl of a suspension of hepatocytes in the appropriate buffer to 600 μl of buffer containing the corresponding substrate. The concentration of the cyclosporines used varied between 0.1 and 10 μM dependent on the extreme low solubility in aqueous solutions, the bile acid concentration was from 0.1 to 500 μM. Cyclosporines were added to the incubation media from stock solutions of ethanol or DMSO to give a final solvent concentration not exceeding 1% (v/v). Thereby, 50 nM labeled drug and serial dilutions of unlabeled drug were used. There was no difference in the uptake between the diluted and the concentrated radioactive labeled compounds. The solvents were also added in reference measurements without cyclosporine. Control experiments (bile acid uptake measurements) showed no difference in uptake rates. Cyclosporine dissolving agents such as cremophor were not used because of its membrane affecting character and because several studies showed a significant decrease of membrane fluidity in the presence of Cremophor (Chervinsky *et al.*, 1993; Fjallskog *et al.*, 1994; Dudeja *et al.*, 1995). The cell-incorporated radioactivity was determined by liquid scintillation counting.

Initial rates of uptake into isolated hepatocytes were calculated from the slopes in the linear range by linear regression analysis, considering only data points within the first 60 sec of uptake (15-sec intervals). The kinetic parameters were analysed by the nonlinear least-square regression analysis program enzfitter 1.05 (Elsevier-Biosoft, Cambridge, UK). The resulting kinetic data are reported as means ± S.E. Statistic differences were determined by use of the appropriate paired or unpaired *t* test.

Determination of cellular cyclosporine concentrations. The amount of cell associated cyclosporine was determined by using a Sandimmune RIA kit from Sandoz Pharma AG, Basle, Switzerland with a nonspecific monoclonal antibody. Cells were washed twice with phosphate-buffered saline, scraped off the plates and supplemented with 3 ml phosphate-buffered saline buffer. A total of 50 μl of the cellular suspensions was added to 950 μl of methanol and subsequently centrifuged at $10{,}000 \times g$ for 15 min. A total of 50 μl of the supernatants was subjected to RIA analysis.

Determination of enzyme activities. The activities of ALT and AST were determined by means of an automated test using the Reflotron system (Boehringer, Mannheim, FRG) following the assay as described by (Deneke and Rittersdorf, 1984, 1985). In both assays the determination of pyruvate by pyruvate-oxidase was followed by formation of acetylphosphate and H_2O_2. In the coupled indicator reaction 4-(4-dimethylaminophenyl)-5-methyl-2-(3.5-di-1-butyl-4-hydroxyphenyl)imidazol-dihydrochloride was used as chromogenic dye.

LDH activity was measured by a photometric assay with the test kit Merck-1-TestR LDH 3349 (Merck, Darmstadt, FRG). For control preparations LDH activity in the cell supernatants after 20 hr of incubation ranged between 300 to 400 U/liter.

Protein determination. Protein concentrations were determined according to Bradford (1976). Bovine serum albumin was used as standard.

Statistic analysis. First, a two-way or three-way analysis of variance was done, depending on whether or not data were obtained from different animals and whether a second independent variable was varied in addition to the first independent variable (Scheffe, 1959). An interaction term between two independent variables was fitted if it was significant. The residuals were subjected to a formal test of normality; if this test came out significant, a quantile plot was used to decide whether there were one or more outlieers. If so, they were deleted and the procedure was repeated. If residuals were still nonnormal, it was assumed that variances were unequal. Second, if residuals turned out to be normally distributed, a Dunnett test was used to investigate the dependence of the response on either CyA or SDZ IMM 125 (Dunnett, 1955, 1980). The Dunnett test compares every treated group to the control group, where the control and treated groups consist of observations with the independent variable set to zero or a constant nonzero value, respectively. If the independent variable had only two levels, a *t* test was used rather than the Dunnett test. If the animal term was significant in the model fitted first, the paired *t* test was use, otherwise the two sample *t* test with equal variances was used. If the variances were unequal (according to the criterion mentioned above), the Dunnett test was replaced with a set of two sample *t* tests with unequal, with Bonferroni correction for the multiple significance level (Satterthwaite, 1946). No nonparametric statistics (*e.g.*, Kruskal-Wallis test) have been performed because it was believed that the data were normally distributed even if variances were not equal, and that the small size of the data set did not allow a successful nonparametric investigation. If two independent variables were present, this procedure was performed independently for every level of the other independent variable. The SAS software was used for the computations (Cary, 1989).

Results

CyA-induced changes in physicochemical membrane properties. The lipophilicity of the two cyclosporines was determined by measuring the partition coefficient between liposomal membranes and buffer. The partition coefficients for CyA and SDZ IMM 125 were 4034 ± 521 and 533 ± 58, respectively (means ± S.D., $n = 20$), indicating a significantly higher lipophilicity of CyA compared to SDZ IMM 125. In addition, liposomal membrane anisotropy was determined in the presence of both compounds. In the steady-state depolarisation fluorescence studies, DOPC liposomes were incubated with increasing amounts of CyA or SDZ IMM 125 in the concentration range between 0.5 to 10 μM. Both cyclosporines caused a concentration-dependent decrease of anisotropy (fig. 1), indicating that they both had a membrane fluidising effect. The effect was more pronounced for CyA (decrease of 46 *vs.* 25% for SDZ IMM 125 measured at 25°C at a lipid drug ratio of 13.1), suggesting that at a given concentration the amount of CyA inserted into the lipid membranes was greater than that of SDZ IMM 125. This result correlated very well with the different lipophilicity of both compounds. Furthermore, Na^+ and Ca^{++} -fluxes across liposomal membrane were determined. The permeability of liposomal membranes for Na^+- and Ca^{++}-ions increased in a dose- and time-dependent manner in the presence of the cyclosporines as shown in figure 2. The diffusion of Na^+ and Ca^{++} was significantly greater in the presence of SDZ IMM 125 than in the presence of CyA, which suggested that SDZ IMM 125 had a greater ionophoric activity than CyA.

Cellular uptake studies. The uptake of cyclosporines into suspensions of freshly isolated hepatocytes was determined in the concentration range from 0.1 to 10 μM (fig. 3). Higher concentrations resulted in a visible precipitation of the compounds due to their highly lipophilic character. After a very rapid concentration dependent absorption to the cells within the first 2 to 3 sec, the uptake was linear over the whole time interval of investigation (up to 5 min). The dependency of the initial rates of uptake upon increasing concentrations was linear for both compounds indicating nonsaturable uptake by passive diffusion. This result was supported by further uptake studies, which showed that uptake was not Na^+-dependent, not temperature dependent

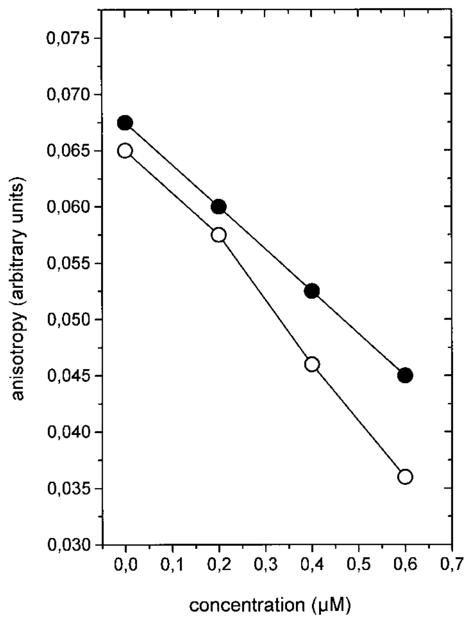

Fig. 1. Steady-state fluorescence anisotropy of DOPC liposomes incubated with different amounts of cyclosporines. Liposomes (2 mg phospholipid/3 ml buffer 50 mM NaCl, 50 mM KCl, 20 mM Tris; pH 7.4) were preincubated with DPH (molar ratio phospholipid: DPH = 200:1) for about 1 hr. Cyclosporines were added from a 100 mM stock solution in ethanol. Measurements (excitation 336 nm, emission 430 nm, 10-sec integration, 3-nm slit width) were taken at 25°C. Ethanol solution without cyclosporines did not show any significant changes in anisotropy. Decrease of anisotropy for CyA (○) and SDZ IMM-125 (●).

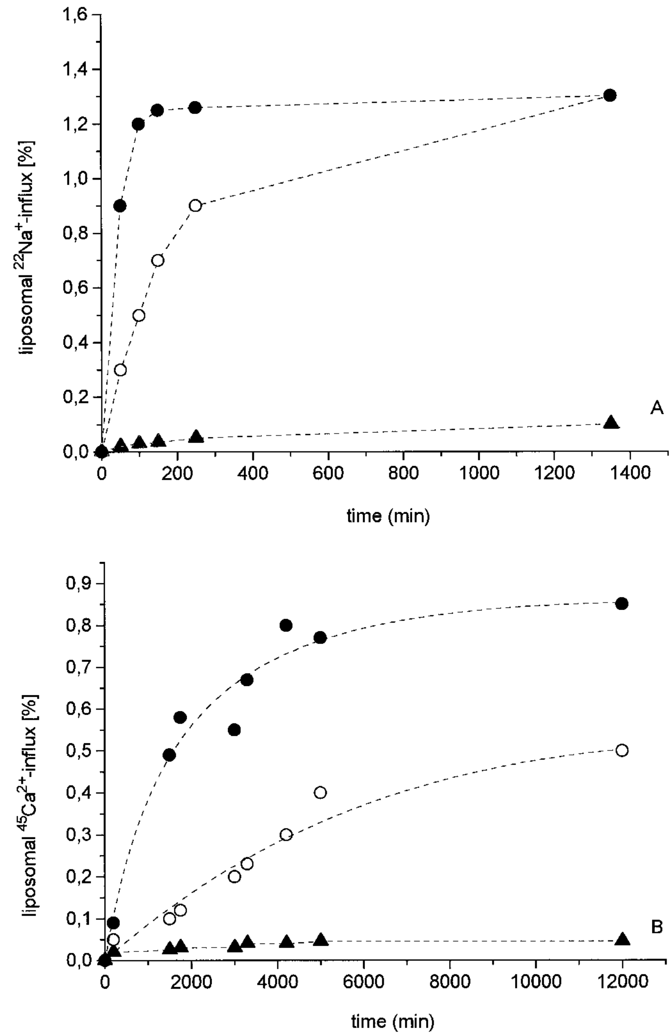

Fig. 2. A, Time-dependent Na^{+}-flux measurements with liposomes loaded with 480 μM CyA (○) or SDZ IMM 125 (●); ▲ = blank. B, Time-dependent Ca^{++}-flux measurements with liposomes loaded with 500 μM CyA (○) or SDZ IMM 125 (●); ▲ = blank. The given concentrations of CyA and SDZ IMM 125 are concentrations in the total lipid suspension; the concentrations in the aequous solution were approximately 20 μM (n = 3; S.D. are covered by the symbols).

and could not be inhibited by the bile salt cholyltaurine in the concentrations up to 100 μM (fig. 3). The diffusion coefficient of CyA was $0.50 \pm 0.02 \times 10^{-4}$ liter $\times$ min^{-1} $\times$ mg^{-1} protein, the diffusion coefficient of SDZ IMM 125 was $0.28 \pm 0.01 \times 10^{-4}$ liter $\times$ min^{-1} $\times$ mg^{-1} protein.

The cell-associated amounts of CyA and SDZ IMM 125 were also determined in hepatocyte monolayers incubated for 24 hr with concentrations of 1, 5, 10, 25 and 50 μM by means of a CyA RIA. For both compounds a dose-dependent increase in cellular concentrations was found (fig. 4). At all medium concentrations, the amount of cell-associated cyclosporine was statistically significantly higher for CyA than for SDZ IMM 125. The ratio between CyA and SDZ IMM 125 was about 2.

Inhibition of bile acid uptake and bile flow. The inhibition of bile acid uptake into hepatocytes might be part of the mechanisms, by which cyclosporines cause cholestasis. Thus, the transport of cholyltaurine into freshly isolated rat hepatocytes was determined by incubation of the cells with increasing bile acid concentrations and subsequent measurement of time dependent, cell-incorporated radioactivity. Transport into the cells exhibited saturability (fig. 5a). When the uptake of cholyltaurine was measured in the presence of increasing concentrations of CyA or SDZ IMM 125 an inhibitory effect on the bile acid transport rates dependent on the concentration of the respective cyclosporine was seen. Graphical analysis in the J/A-*vs.*-J diagram (fig. 5b) revealed an apparently competitive type of inhibition. The same result was obtained, when cholate was used as radioactive labeled substrate. The isolated perfused rat liver is an intact organ system, where the structural and functional integrity is maintained for several hours. This investigative model is suitable for studying transcellular bile acid transport including secretion processes. The cholestatic potential of a compound can easily be evaluated by bile flow determinations. The influence of both cyclosporines on bile flow is illustrated in figure 6. Both cyclosporins caused a dose- and time-dependent decrease of bile flow compared to untreated controls. Maximum inhibitory effects were achieved after 30 min of perfusion. SDZ IMM 125 was more potent in decreasing the bile flow compared to CyA, but significant differences between both derivatives were only seen at the concentrations of 50 μM. At that concentration and after 30 minutes of perfusion with CyA bile flow decreased 25% *vs.* controls, whereas the decrease after perfusion with SDZ IMM 125 was 50%. This result suggests that SDZ IMM 125 has a greater cholestatic potential compared to CyA.

Cyclosporine-induced hepatic enzyme release. Cytotoxicity of CyA and SDZ IMM 125 was determined in 24 hr primary hepatocyte mono-layer cultures by measuring the release of LDH into the cell culture medium. Concentrations

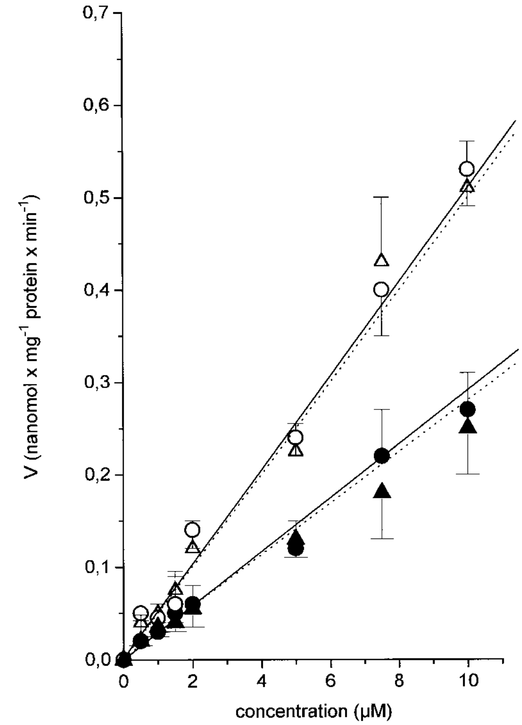

Fig. 3. Concentration dependent uptake of cyclosporines into freshly isolated hepatocytes (○) CyA, (△) CyA in presence of 100 µM cholyltaurine; (●) SDZ IMM 125; (▲) SDZ IMM 125 in presence of 100 µM cholyltaurine (n = 6; means ± S.E.).

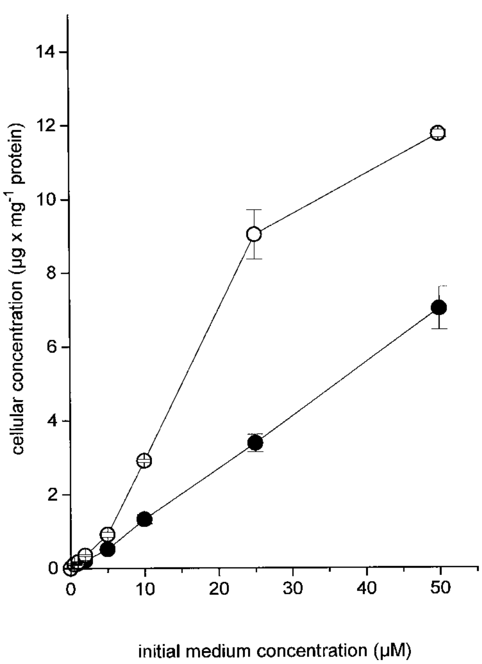

Fig. 4. Concentration dependent intracellular accumulation of CyA (○) and SDZ IMM 125 (●) in primary rat hepatocyte cultures after 24 hr incubation (n = 6; means ± S.E.).

of 10 and 25 µM of either cyclosporine did not induce a statistically significant increase of LDH values compared to control (fig. 7). At concentrations of 50 µM of the respective cyclosporine the LDH values in the culture medium were significantly increased. SDZ IMM 125 was statistically significant more toxic than CyA. Time-dependency of the cyclosporine toxicity was determined at concentrations of 50 µM. CyA and SDZ IMM 125 caused a time-dependent increase of extracellular LDH activity, starting onset after 7 hr (fig. 8). Differences between the two derivatives were observed after 12-hr incubation time. Thereby, SDZ IMM 125-induced LDH-release was statistically significant higher than that induced by CyA.

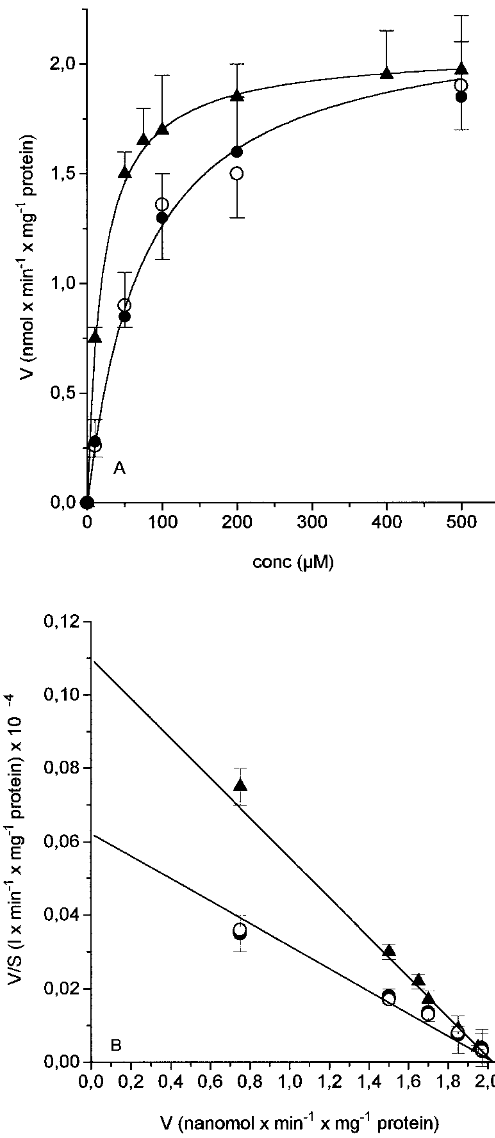

Fig. 5. A, Concentration-dependent uptake of cholytaurine into freshly isolated rat hepatocytes: (▲) control; (○) in presence of 10 µM CyA; (●) in presence of 10 µM SDZ IMM 125 (n = 6; means ± S.E.). B, Eadie-Scatchard diagram indicating competitive inhibition.

Cytotoxic effects of the cyclosporines were also investigated in isolated perfused rat liver by determination of LDH release into the perfusate. During the first 3 hr of perfusion the level of LDH activity in the perfusate of control livers was constant, indicating organ viability over that time. The criterion for toxicity was set to the time points, when 2-, 5- or 10-fold of that baseline LDH values was reached. For controls multiples of the baseline were reached between 190, 210 and 225 min of perfusion (fig. 9). With cyclosporine concentrations of less than 25 µM no statistically significant differences between the cyclosporines and the respective controls

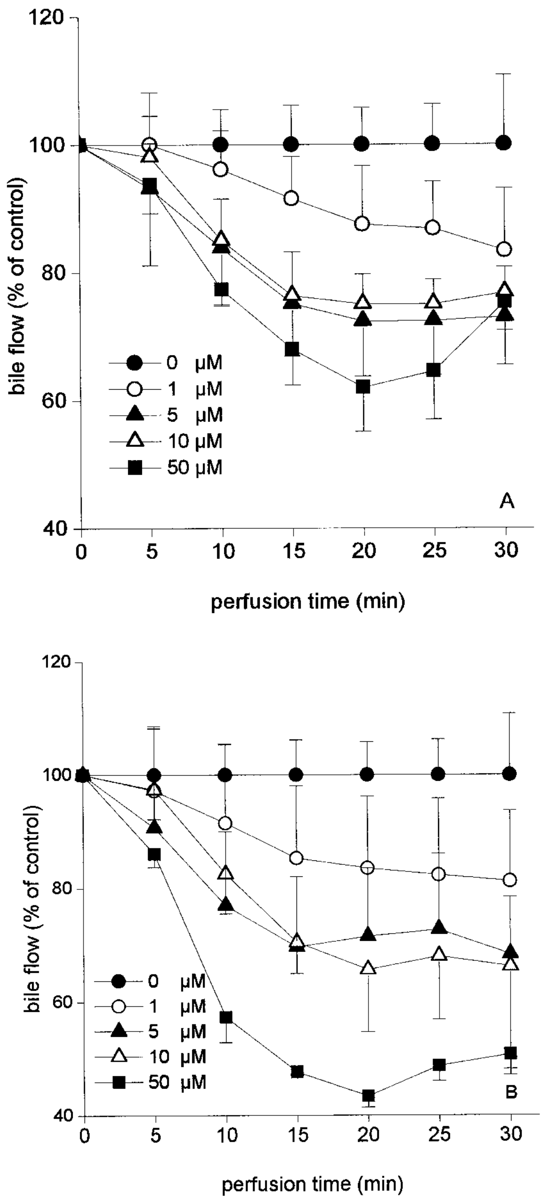

Fig. 6. Bile flow of isolated perfused rat livers perfused with CyA (A) or SDZ IMM 125 (B) at different perfusate concentrations of either cyclosporine (n = 5-6; means ± S.D.). Significant differences ($P < .05$) compared to time zero were observed at all concentrations and at all time points greater than 10 min, except at 1 μM CyA or SDZ IMM 125. Significant differences ($P < .05$) between CyA and SDZ IMM 125 were observed at 50 μM at all values measured after 5 min.

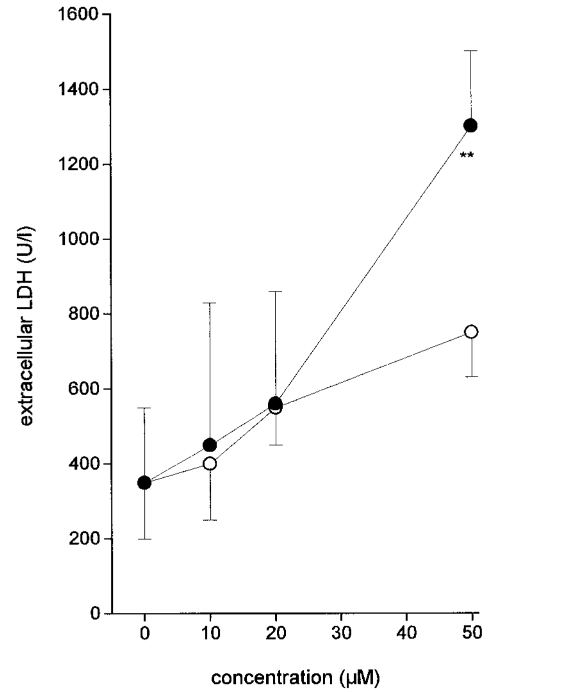

Fig. 7. Effect of CyA (○) and SDZ IMM 125 (●) on the release of LDH in primary hepatocyte 24-hr cultures (n = 6; means ± S.E.; Statistically significant differences between CyA and SDZ IMM 125 are indicated by **($P < .01$).

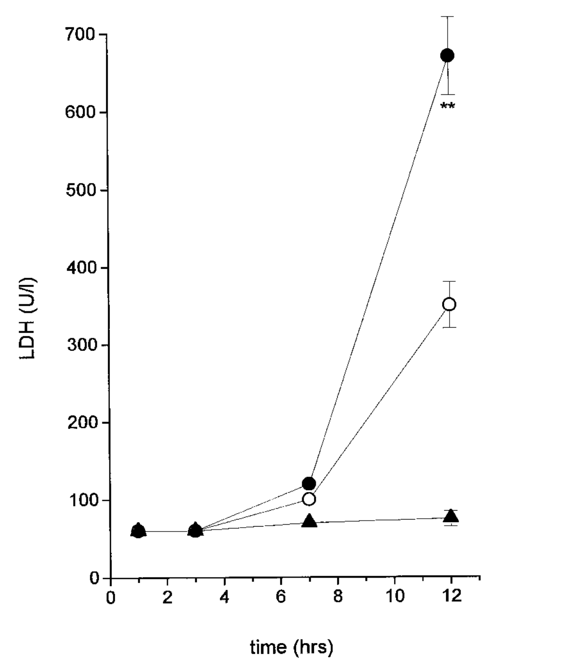

Fig. 8. Time-dependent LDH release into the cell culture medium after incubation of hepatocyte primary cultures without cyclosporine (▲) or with 50 μM CyA (○) or 50 μM SDZ IMM 125 (●) (n = 6; means ± S.E.; Statistically significant difference between CyA and SDZ IMM 125 is indicated by ** ($P < .01$). Values obtained after 12 hr of cyclosporines were all statistical significant from untreated controls ($P < .001$).

were found. However, when the cyclosporine perfusion medium concentration was 50 μM, LDH activity started to increase in the perfusate much earlier than in control livers, indicating cytotoxicity. Based on the multiple baseline criterion CyA cytotoxicity was reached 165, 175 and 185 min after starting the perfusion. This effect was even more pronounced after perfusing with SDZ IMM 125. The onset of LDH-release shifted towards 120 min (2-fold baseline), 145 min (5-fold) and 160 min (10-fold baseline), which was significantly earlier than with CyA. These data confirm the greater cytotoxic potential of SDZ IMM 125 compared to CyA, which was found in the 24 hr hepatocyte primary culture.

ALT and AST activities were determined in parallel to the LDH activity in the perfusate of the perfused livers (data not shown). There was a good correlation between LDH release and release of ALT and AST as parameter of cytotoxicity. The correlation coefficients were calculated by linear regression from individual values obtained from all performed perfusions. The correlation coefficient of LDH and AST was r =

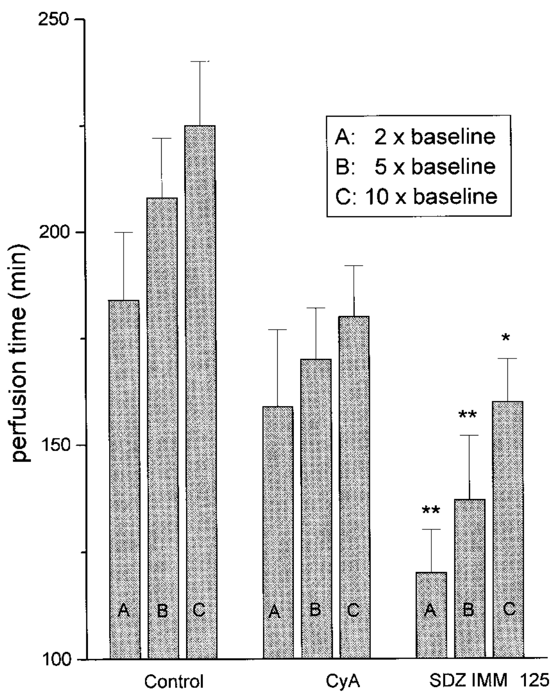

Fig. 9. Release of LDH from isolated perfused rat liver into the perfusion medium. Initial concentrations of CyA and SDZ IMM 125 in the perfusate were 50 μM (n = 5; means ± S.E.). Statistical significant difference between the respective SDZ IMM 125 and CyA groups are indicated by *P < .05 and **P < .01. All values were statistically significant different compared to the respective controls (P < 0.05) except group CyA/A.

0.891 (P < .05), that of LDH and ALT was 0.948 (P < .01), respectively.

Discussion

In preclinical toxicological evaluations the novel derivative SDZ IMM 125 was found to be superior to CyA mainly because of less renal side effects (Donatsch *et al.*, 1992; Hiestand *et al.*, 1993). However, in humans there was clear evidence for liver intolerability of SDZ IMM 125 resulting in significant increases of the liver specific serum transaminases ALT and AST (Witkamp *et al.*, 1995). The magnitude and the incidence of such effects were never observed after administration of CyA, neither in healthy volunteers nor in transplant patients. However, CyA was found to increase serum bile acid levels in animal and man, indicating a cholestatic potential of the drug (Ellis *et al.*, 1986; Stone *et al.*, 1987; Azer and Stacey, 1991). A cholestatic potential of SDZ IMM 125 is so far unknown.

In our study we tried to measure the hepatic side effects of SDZ IMM 125 by comparing its acute cholestatic and cytotoxic effects with those of CyA in different liver specific *in vitro* models. The models that were selected for this purpose consisted of systems with different degree of complexibility ranging from model membranes to isolated cell suspensions, containing only one cell type, until to a whole perfused organ, which is nearly completely maintained in its whole tissue architecture containing all types of naturally occurring cells in the intact liver.

By means of the present *in vitro* experiments it was possible to mimic both the cholestatic effects of both compounds, as well as their response concerning liver enzyme release, which was found after *in vivo* treatment. For the determination of the cytotoxic potential, the cyclosporines were investigated in the isolated perfused rat liver and in the hepatocyte primary cultures. The increase of LDH activity in the perfusate was selected as an indicator of cytotoxicity because of the good correlation between the liver specific enzymes ALT, AST and the LDH activity. Because LDH was a more sensitive parameter compared to ALT and AST, we used this enzyme for all experimental investigations.

In our *in vitro* experiments relatively high concentrations of cyclosporins up to 50 μM have been used to assess short-term toxicity. Taking into consideration that in subchronic rat studies at daily doses of up to 50 mg/kg blood peak levels of 10 to 15 μM had been observed (Donatsch *et al.*, 1992, Wolf *et al.*, 1994, 1997), it can be assumed, that due to the high lipophilicity of cyclosporines an even higher local accumulation in the liver occurs, justifying the used experimental conditions.

In the isolated perfused liver the onset of LDH release started much earlier (120 min) with SDZ IMM 125 than with CyA. (165 min). From this, it was concluded that SDZ IMM 125 is more toxic than CyA. In the primary hepatocyte culture LDH leakage after the SDZ IMM 125 treatment was also greater compared to that of CyA. Taken the results of both systems together, they suggest that SDZ IMM 125 has a higher intrinsic cytotoxic potential than CyA. Our *in vitro* data are in agreement with a very recent multiple dose trial in patients with severe psoriasis receiving up to 400 mg SDZ IMM 125. In this study changes in liver function were the main adverse events. A clear-cut dose-dependent increase of some liver enzymes, mainly of AST was observed, which was reversible after treatment had stopped (Witkamp *et al.*, 1995).

Elevated serum bile acid levels after CyA treatment are indicators of the interaction of the drug with the hepatic bile acid transport and cholestasis. However, the available data about the type of interaction are ambiguous. To evaluate the cholestatic potential of both compounds, we investigated the interaction of the two cyclosporines with hepatic bile acid uptake in freshly isolated hepatocytes. In our study, both cyclosporines inhibited cholyltaurine uptake apparently competitively to a similar extent. A competitive inhibition of CyA has recently been reported in a study with rat hepatocyte basolateral plasma membranes and hepatocyte primary cultures (Moseley *et al.*, 1990; Kukongviriyapan and Stacey, 1988). However, because there was no evidence for active uptake, the apparently competitive inhibition might be the result of an unspecific binding to the bile acid transporters as it was suggested earlier for a proposed cholate carrier (Ziegler and Frimmer, 1986). Own preliminary photoaffinity labeling studies with a photolabile cyclosporine derivative gave no evidence for a specific binding to the basolateral bile acid carrier proteins (data not shown). This is in clear contrast to previous observation of a specific labeling of membrane proteins by photolabile bile acid derivatives (Ruetz *et al.*, 1987). Therefore, the inhibition of bile acid uptake seems to be rather a sign of a general membrane alteration than a specific carrier-related process. This hypothesis is further supported by the a very rapid incorporation of the cyclosporines into pure phospholipid membranes and by the rapid exchange of CyA between blood cells and liposomes (Fahr *et al.*, 1995). In addition, other studies with human lymphocytes and liposomal preparations demonstrated the absence of specific membrane binding sites for CyA (LeGrue *et al.*, 1983) and indicated a simple partitioning into the lipid phase. Experiments with intact proximal tubules also indicate that in the kidney cyclosporines also enter tubular cells from the blood by passive diffusion (Schramm *et al.*, 1995).

The effects of the two cyclosporines on bile flow was studied in the isolated perfused rat liver. In contrast to hepatocytes in suspension and in monolayer culture, the isolated perfused rat liver maintains its polar functions of bile acid uptake and secretion for several hours. Perfusion of the liver with medium containing the cyclosporines immediately resulted in a decrease in bile flow, which was higher with SDZ IMM 125 than with CyA at equimolar conditions. This demonstrates, that in contrast to the inhibition of uptake of bile acids by the two cyclosporines in freshly isolated hepatocytes, which was nearly equal, bile secretion seems to be inhibited much more by SDZ IMM 125. These results suggest that the greater cholestatic potential of SDZ IMM 125 is mainly due to the inhibition of the bile flow and not as it was initially assumed by the inhibition of the bile acid uptake.

The mechanisms leading to the stronger cholestatic and greater cytotoxic activity of SDZ IMM 125, compared to CyA are not fully understood. There might be some relationship between both events and their physical-chemical properties. The two cyclosporines were considerably different with regard to their lipophilicity. By means of their partition coefficients between liposomes and buffer, it was clearly shown that CyA was more lipophilic than SDZ IMM 125. This property was also reflected by their effects on membrane anisotropy, which was greater for CyA than for SDZ IMM 125, suggesting that the amount of CyA, which was incorporated into the membranes was more than that of SDZ IMM 125. The different lipophilicity of the two cyclosporines might have an impact on their cellular uptake and the final cellular exposure. The determination of the cellular concentrations of the cyclosporines revealed, that at equal medium concentrations about twice as much CyA was associated to the cells as SDZ IMM 125. Considering the lower cellular exposure of SDZ IMM 125 in comparison to CyA this suggests the higher cytotoxic and cholestatic potential of SDZ IMM 125.

The present studies showed that both cyclosporines increased the membrane permeability for Na^+ and Ca^{++} ions, which, however, was most dramatically increased by SDZ IMM 125. Changes in the intracellular ion homeostasis might directly contribute by disturbing the transmembrane potential and cellular ion gradients. Similar findings were obtained by other groups. The K^+-efflux from preloaded human lymphocytes was increased by CyA at nontoxic concentrations in a dose-dependent manner and the membrane potential of lymphocytes was significantly reduced (Damjanovich *et al.*, 1987; Matyus *et al.*, 1986). Although not quantitatively, such a mechanism might contribute to the decrease of the hepatic bile acid uptake for both cyclosporines.

Increased intracellular Ca^{++} contents may directly cause toxicity. It is very well known that intracellular free Ca^{++} can serve as a mediator of cytotoxicity by activating catabolic pathways by which important cellular macromolecules like proteins, lipids and nucleic acids are degraded (Orrenius, 1993; Kehrer, 1993). Because increased liposomal permeability for Ca^{++} ions goes in parallel with the cytotoxic potential of SDZ IMM 125 and CyA in hepatocytes, increased intracellular Ca^{++} concentrations might by one favorite mechanism of cytotoxicity under our present experimental conditions.

In summary, the results obtained with our experimental *in vitro* models, suggest that SDZ IMM 125 exhibited a greater intrinsic potential than CyA to impair liver functions, resulting in cholestasis and liver cell damage. The studies showed that it is possible to determine the potential intrinsic cytotoxicity and cholestatic effects of new cyclosporine developments by using our investigated *in vitro* methods, the isolated perfused rat liver to determine both effects, whereas the hepatocyte primary culture can only be used to determine compound-induced liver cell damage. The results imply that for therapeutical reasons and to circumvent liver adverse side effects of SDZ IMM 125, other administration schedules than the oral route should be envisaged, because by oral administration too high drug concentrations are achieved via the portal vein, which directly might damage the liver. However, the clinical relevance of these findings depends very strictly from the individual pharmacokinetic property of the respective new cyclosporine.

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