Biochemical Pharmacology, Vol. 39, No. 2, pp. 257–266, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

EFFECT OF ENZYME INDUCTION ON SANDIMMUN® (CYCLOSPORIN A) BIOTRANSFORMATION AND HEPATOTOXICITY IN CULTURED RAT HEPATOCYTES AND *IN VIVO*

PATRICK BOUIS, JEAN-FRANCOIS BROUILLARD, VOLKER FISCHER, PETER DONATSCH and URS A. BOELSTERLI*†

Department of Toxicology, Drug Safety Assessment, Sandoz Ltd, CH-4002 Basel, Switzerland and *Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, CH-8603 Schwerzenbach, Switzerland

(*Received* 27 *April* 1989; *accepted* 1 *August* 1989)

Abstract—This study was designed to examine the relationship between the extent of Sandimmun® (cyclosporin A, SIM) metabolism and SIM-induced hepatotoxicity both *in vivo* and in primary cultures of rat hepatocytes. *Firstly*, SIM (50 mg/kg p.o.) was administered daily to male Wistar rats for 10 days with or without co-administration of Aroclor 1254. SIM-induced hepatotoxicity appeared after 4 days of treatment and was enhanced after 10 administrations of SIM. Total plasma proteins were decreased and hyperbilirubinemia as well as increased levels of plasma bile salts were prominent. Aroclor 1254 stimulated total hepatic cytochrome P-450 3.7-fold, and markedly increased the rate of SIM metabolism and plasma elimination as determined by both HPLC and RIA techniques. However, this induction did not change the degree of SIM-induced hepatotoxicity. *Secondly*, short-term cultures of hepatocytes obtained from normal rats and from rats pretreated with either Aroclor 1254 or dexamethasone, a specific inducer of the cytochrome P-450 III gene family responsible for the formation of the primary SIM metabolites M1, M17 and M21, were incubated with various concentrations of SIM for up to 17 hr. At 1 μM SIM, both inducers greatly increased the rate of SIM metabolism *in vitro*, producing, however, different metabolite patterns. In the hepatocyte cultures, SIM inhibited the incorporation of amino acids into proteins. In addition, a small fraction of [3H]-labeled SIM was covalently bound to hepatocellular macromolecules. Although the fraction of covalently bound SIM was markedly increased in cells from dexamethasone-treated rats, the degree of inhibition of hepatocellular protein synthesis was not changed in cells from induced rats. In contrast to SIM-induced nephrotoxicity, these results suggest that increased rates of SIM biotransformation by inducers of drug metabolism are not associated with an attenuation of hepatotoxicity both *in vivo* and *in vitro*.

The immunosuppressive cyclic oligopeptide Sandimmun® (SIM, cyclosporin A)‡ is extensively used in organ transplantation and autoimmune disorders. Its therapeutic use is, however, often limited by side effects including nephrotoxicity and hepatic dysfunction [1–8]. Clinically, the functional impairment of the liver is manifested by increased plasma levels of bilirubin and bile acids, accompanied by mild increases in plasma alkaline phosphatase and transaminase activities [6–11]. Mechanisms of SIM-induced hepatotoxicity have been investigated in several animal models, among which the rat has proved to be useful in characterizing the functional changes like cholestasis and impairment of protein synthesis [12, 13]. In particular, primary cultures of rat hepatocytes have been successfully used to define some of the cellular pathomechanisms involved in SIM cytotoxicity [14–16].

SIM is largely metabolized by the hepatic cytochrome P-450-dependent monooxygenases [17–19] and primarily eliminated via the bile [20]. All of the major metabolites identified so far (mono- and dihydroxylated, N-demethylated) retain the cyclic oligopeptide structure [18], but the intrinsic toxicity of these metabolites is largely unknown. Several lines of evidence suggest, however, that the toxic effects associated with SIM administration may be due to the parent compound rather than to metabolites. First, direct administration of one of the major metabolites M17 to rats *in vivo* [21] or exposure of rat hepatocytes to this metabolite *in vitro* [22] produced much smaller signs of toxicity than equimolar amounts of the parent compound. Second, induction of hepatic drug metabolism with phenobarbital or Aroclor 1254 in rats reduced SIM-induced structural and functional changes in the kidney, suggesting that increased rates of SIM biotransformation and elimination ameliorated the drug's nephrotoxic potential [23–25]. Finally, there is evidence from clinical studies that inhibitors of hepatic drug metabolism may potentiate SIM nephrotoxicity, whereas enzyme inducers reduced SIM plasma concentrations and SIM-associated nephrotoxicity [25].

† Author to whom all correspondence should be addressed at: Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, Schorenstrasse 16, CH-8603 Schwerzenbach, Switzerland.

‡ Abbreviations used: ACN, acetonitrile; ALT, alanine aminotransferase; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; FCS, fetal calf serum; LDH, lactate dehydrogenase; 3-MC, 3-methylcholanthrene; PB, phenobarbital; SIM, Sandimmun® (cyclosporin A); TCA, trichloroacetic acid; TMAHS, tetramethylammonium-hydrogen sulfate.

The effects of increased rates of SIM biotransformation on the extent of SIM-induced functional liver changes have not yet been studied. SIM is highly accumulated in the hepatic parenchymal cells, and only slowly metabolized. Furthermore, virtually all of the metabolites are excreted via the biliary pathway, but the quantitative profile in the bile differs from that in blood or urine [26]. In view of the central role of the liver for SIM metabolism and excretion, the present study was designed to examine interrelationships between increased rates of SIM biotransformation and SIM-associated hepatotoxicity both *in vivo* and in primary cultures of rat hepatocytes. For this purpose, rats were pretreated with two inducers of drug metabolism, and the quantitative and qualitative profile of SIM biotransformation was correlated with endpoints indicative of hepatic toxicity.

MATERIALS AND METHODS

In vivo *experiments*

Treatment of animals. To assess the effects of induction of xenobiotic biotransformation on SIM metabolism and hepatotoxicity, male Wistar rats (Kfm:WIST, 180–220 g body wt, Kleintierfarm Madörin, Füllinsdorf, Switzerland) were injected intraperitoneally with 100 mg/kg of Aroclor 1254 in olive oil or olive oil alone on days −2, 1, 4 and 7 of the experiment. During this treatment and after the first administration of inducer, SIM or Cremophor vehicle was given orally (50 mg/kg/day) for 10 days. *In vivo* pretreatment of animals with intraperitoneal administration of either Aroclor 1254 or dexamethasone (100 mg/kg, both dissolved in olive oil) for *in vitro* studies was performed as described above, with a total of two administrations 6 and 3 days before the isolation and subsequent culture of the hepatocytes.

Plasma biochemistry. After five and ten daily administrations of SIM, retroorbital blood samples were taken in heparinized tubes and centrifuged for 15 min (2000 *g* at 4°), both 5 and 24 hr after the last SIM administration. Plasma supernatants were then divided for biochemical analysis, using an Olympus eppendorf model Eris Analyzer 6170 automatized system, and for the determination of SIM plasma concentration.

Determination of SIM and SIM metabolites plasma concentrations. (i) High performance liquid chromatography (HPLC): the parent compound SIM was determined using an HPLC method. Plasma (0.2 ml) was rendered alkaline by adding 0.5 ml of 0.1 M Merck-Titrisol borate buffer, pH 11, and extracted with 1.5 ml hexane containing 5% isopropyl alcohol (v/v). A fraction (1.2 ml) of the organic phase was then transferred into another tube and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 150 μl of acetonitrile (ACN) and 0.1% aqueous solution of tetramethylammoniumhydrogen sulfate (TMAHS) (65/35, v/v). An aliquot of 120 μl was then injected on a reverse phase RP 8 Brownlee 4.6 × 30 mm, 10 μm precolumn, linked with a reverse phase RP 18 4.6 × 100 mm, 5 μm column, and maintained at 65°. The elution of SIM was performed with the following gradient system: 6 min ACN 50%, 0.1% TMAHS 50% (equilibrium), 5 min linear gradient from 50 to 65% ACN, 5 min ACN 65%, 2 min ACN 75%, with a flow rate of 1.8 ml/min. The detection was performed using a Gilson 116 UV detector set at 210 nm. Under these conditions, SIM was eluted with a retention time of 13.01 min with a detection limit of 50 ng/ml and an extraction efficiency of 80%. (ii) Radioimmunoassay (RIA): the cyclosporin RIA-kit containing polyclonal antibodies allowed the quantitation of both SIM and immuno-cross-reacting metabolites [27]. Aliquots of plasma were extracted with ether, evaporated under a stream of nitrogen, and redissolved in RIA buffer.

Liver cytochrome P-450 content and activities of ethoxycoumarin-O-deethylase (ECOD) and cytochrome P-450 reductase. At the end of the treatment the rats were anesthetized with an intraperitoneal administration of 60 mg/kg pentobarbital. The livers were then rapidly perfused *in situ* with cold saline in order to remove the blood, and a piece of the left lobe was homogenized with a motor driven glass–teflon homogenizer (1000 rpm, 10 strokes) in cold 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.2% Emulgen 913 (33 mg liver/ml buffer). The homogenates were then centrifuged at 8700 *g* for 8 min at 4°. Cytochrome P-450 content was determined in the supernatant according to Omura and Sato [28] using a single beam Beckman DU-7 spectrophotometer. Since Emulgen 913 inhibited ethoxycoumarin-*O*-deethylase activity, a separate piece of liver was homogenized as described above, in cold 0.1 M phosphate and 0.15 M KCl buffer, pH 7.4 (200 mg liver/ml buffer). After centrifugation, aliquots of the supernatants were assayed for ECOD activity and cytochrome P-450 reductase. ECOD activity was assessed using 500 μM ethoxycoumarin, as described by Greenlee and Poland [29], except that the reaction mixtures were bubbled for 10 sec with oxygen prior to the incubation. Cytochrome P-450 reductase activity was determined according to Falzon *et al.* [30].

In vitro *experiments*

Primary rat hepatocyte cultures. Following *in vivo* enzyme induction with either Aroclor 1254 or dexamethasone (see treatment of animals), or after treatment with the vehicle alone, the isolation of the hepatocytes was performed as described earlier [22]. Briefly, the liver was perfused *in situ* with Ca^{2+}– and Mg^{2+}–free Hank's bicarbonate buffer for 5 min at 37°. The liver was then removed and perfused with a recycling oxygenated Hank's medium containing 5 mM Ca^{2+} and 100 units/ml collagenase (type I CLS, Biochrom). Following mechanical dissociation, the cells were suspended in Williams' medium E (WME), passed through a Cellector tissue sieve (Bellco, Vineland, NJ) and allowed to sediment (15 min at 4°). The pellet was then washed twice by resuspending the cell pellet in fresh medium. The viability of the hepatocytes, assessed by trypan blue exclusion, was > 90%. The cells were seeded into 60 mm culture dishes (Primaria, Beckton-Dickinson, Oxnard, CA) in 3 ml WME supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (0.1 mg/ml), insulin (10^{-7} M), dex-

amethasone (10^{-7} M) and cultured for 3 hr at 37° in a 5% CO_2 atmosphere. The medium was then replaced with fresh WME containing 1.6% FCS, the antibiotics and hormones (supplemented WME), in the presence or absence of SIM.

In vitro *exposure to SIM*

A stock solution of SIM (2 mg/ml DMSO) was prepared on the day of the experiment, directly added to supplemented WME, and the solvent concentration adjusted with DMSO (1.55%, v/v, final concentration) in all culture dishes including the controls. Final SIM concentrations were between 0.1 and 15 μM. The hepatocyte cultures were incubated at 37° in a 5% CO_2 atmosphere.

Assessment of covalent binding of SIM. For the assessment of covalent binding of SIM to hepatic macromolecules, an ethanolic solution of radiolabeled SIM was evaporated under a stream of nitrogen, redissolved in a small volume of DMSO and directly added to supplemented WME (20 μCi [^{3}H]SIM per culture dish). The SIM stock solutions were then added. Following exposure to radiolabeled SIM, the culture media were aspirated and the cell monolayers washed with 3 × 5 ml ice-cold saline. The cells were then scraped off with a rubber spatula in 2 × 1 ml ice-cold double-distilled water and ultrasonicated for 1 min. Aliquots of the homogenates were analysed for total radioactivity of SIM and SIM metabolites. Total protein content was determined according to the method of Bradford [31], using BSA as standard. One mg SIM, dissolved in 100 μl methanol, was added per ml homogenate, to reduce all radioactivity not covalently bound to macromolecules, followed by the addition of 4 ml ice-cold methanol. Precipitate formation was completed after 30 min at 4°. The denaturated macromolecules were filtered on glass filters, and washed with 2 × 5 ml methanol and 2 × 5 ml ethanol. The pellets were transferred into counter vials containing 10 ml Lumagel (Lumac) and vigorously agitated.

Quantitative determination of SIM metabolism. Hepatocyte cultures from untreated or Aroclor 1254-treated rats were exposed to 1 μM or 10 μM SIM containing [^{3}H]SIM as a tracer. After 2 and 17 hr incubation, the culture media were separated from the cells, centrifuged at 200 *g* for 5 min to remove detached cells, and diluted with an equal volume of ethanol. The cell monolayers were washed with 3 × 5 ml ice-cold saline and scraped into 4 ml ethanol/water (1/1, v/v). [^{3}H]SIM and its metabolites were analysed by high performance liquid chromatography. The analytical system consisted of a HP 1090L equipped with a manual injector and a 1 ml sample loop (Hewlett-Packard AG, Switzerland). A guard column and two analytical columns were connected in series (50 × 4.6 mm and 250 × 4.6 mm, 5 μm Supelcosil LC 18, Supelco Inc., Gland, Switzerland). The eluate was monitored for radioactivity (LB 503-detector, Berthold, Wildbad, F.R.G.). The mobile phases consisted of water/methanol, 3/1 (v/v; solvent A) and acetonitrile (solvent B). The proportion of solvent B was 0% from 0 to 2 min, then increased linearly to reach 40% at 4 min, 70% at 84 min and 100% at 90 min. The flow rate was 1 ml/min; the column temperature was 70°. Metabolites were identified according to their retention times. Rat plasma samples containing SIM and its metabolites were used as standards.

Protein biosynthesis. The determination of protein biosynthesis was assessed exactly as described earlier [32]. In summary, after 17 hr incubation of the hepatocytes with 0.5 μCi/dish of a [^{3}H]amino acid mixture in the presence or absence of SIM, medium and cells were separated and proteins were precipitated with trichloroacetic acid (TCA; 10%, w/v). The total of labeled amino acid incorporated into TCA-insoluble proteins of the cells and the medium was determined by liquid scintillation counting.

Statistics. One-way analysis of variance/*F* tests (BBN Software Products Corp., Cambridge, MA) were used for *in vivo* experiments to calculate the significance of differences between results from each group of rats.

Chemicals. Dexamethasone, bovine serum albumin (BSA), bovine insulin and dimethylsulfoxide (DMSO) were purchased from Fluka AG (Buchs Switzerland). Ethoxycoumarin and dithiothreitol were obtained from Sigma Chemical Co. (St Louis, MO) and Emulgen 913 from Kao Co. (Tokyo, Japan). HPLC grade acetonitrile was from Rathburn (Walkerburn, U.K.). Aroclor 1254 was obtained from Analabs Inc. (New Haven, CT). SIM, Cremophor vehicle, and the RIA cyclosporin-kit were provided by Sandoz Ltd (Basel, Switzerland). [^{3}H]SIM was synthesized by Sandoz Ltd (synthesis #706-4, radiochemical purity: 98%) with a specific activity of 14.4 mCi/mg, and [^{3}H]amino acid mixture was from Amersham International (Amersham, U.K.).

RESULTS

In vivo *experiments*

Biochemical indices of hepatotoxicity. The effects of Aroclor 1254 treatment on SIM-induced hepatotoxicity are shown in Table 1. While treatment with Aroclor 1254 had no effect on plasma parameters of hepatic function in comparison with the control group, the 10 oral doses of SIM (50 mg/kg/day) produced a 2.9-fold increase in bilirubin concentration and a moderate reduction of total protein concentration (10%). Total conjugated bile acid concentrations increased by a factor of 2.2 in the plasma samples which were taken 5 hr after the last administration of SIM but decreased thereafter to concentrations similar to those in control rats. Concomitant treatment with Aroclor 1254 and SIM did not modify the hepatotoxicity induced by SIM. The activities of plasmatic ALT in both SIM and SIM + Aroclor 1254 groups remained similar to those in control rats.

SIM concentrations. The measurement of SIM concentrations was assessed by high-performance liquid chromatography and by radioimmunoassay. The former analytical technique measures the concentration of the parent compound, whereas the RIA method, using polyclonal antibodies, enables the determination of both SIM and immuno-cross-reacting SIM metabolites. Table 2 shows that treatment with Aroclor 1254 greatly reduced the trough levels of SIM in plasma by a factor of 3.3 and SIM + metabolites by 3.6 after five oral administrations of

Table 1. Plasma biochemistry after oral administration of SIM in control and Aroclor 1254-induced rats*

Treatment	Bilirubin (μmol/l) 24 hr†	Total proteins (g/l) 24 hr†	Total conjugated bile acids (μmol/l) 5 hr†	Total conjugated bile acids (μmol/l) 24 hr†	ALT (units/l) 24 hr†
Control	2.93 ± 0.60	66.9 ± 0.7	3.43 ± 1.28	4.05 ± 2.47	52.5 ± 28.8
SIM	8.10 ± 3.13‡	60.2 ± 2.1‡	7.60 ± 0.14‡	5.58 ± 2.72	37.3 ± 5.7
Aroclor 1254	2.99 ± 0.15	68.1 ± 2.3	2.75 ± 0.61	3.35 ± 0.58	74.8 ± 11.6
Aroclor 1254 + SIM	6.81 ± 1.76§	55.1 ± 6.0§	7.32 ± 4.18	4.53 ± 1.80	54.3 ± 14.0

* SIM (50 mg/kg/day) or the vehicle (5 ml/kg SIM placebo) was administered for 10 days. Aroclor 1254 (100 mg/kg/day) or the vehicle (1 ml/kg olive oil) was given intraperitoneally 2 days prior to SIM administration and on days 1, 4 and 7 of the treatment. The data represent means ± SD of three rats per group.
† Time after the last application of SIM at which blood samples were taken.
‡ Significant differences from control ($P < 0.05$).
§ Significant differences from Aroclor 1254 ($P < 0.05$).

Table 2. Plasma concentrations (μg/ml) of SIM and SIM metabolites after oral administration of SIM in control and Aroclor 1254-induced rats determined by HPLC or RIA*

Treatment	Method of analysis	Duration of SIM administration: 5 days; Time after the last administration of CsA: 5 hr	5 days; 24 hr	10 days; 5 hr	10 days; 24 hr
SIM	HPLC	5.89 ± 3.20	2.67 ± 0.35	9.93 ± 5.11	5.25 ± 2.14
Aroclor 1254 + SIM	HPLC	6.33 ± 0.55	0.80 ± 0.32†	6.93 ± 4.39	0.90 ± 0.43†
SIM	RIA	10.55 ± 1.34	4.43 ± 1.24	14.05 ± 6.29	5.80 ± 1.98
Aroclor 1254 + SIM	RIA	10.50 ± 2.02	1.23 ± 0.28†	14.63 ± 10.68	1.13 ± 0.56†

* Drug treatment as described in legend to Table 1.
† Significant differences from SIM groups ($P < 0.05$).

Table 3. Cytochrome P-450 content, and activities of cytochrome P-450 reductase and ethoxycoumarin-*O*-deethylase in liver S9 fractions after oral administration of SIM in control and Aroclor 1254-induced rats*

Treatment	Cytochrome P-450 (pmol/mg S9 protein)	Cytochrome P-450 reductase (nmol/min/mg S9 protein)	ECOD (nmol/min/mg S9 protein)
Control	193 ± 9	70.7 ± 9.7	0.48 ± 0.05
SIM	210 ± 20	57.5 ± 7.6	0.42 ± 0.01
Aroclor 1254	705 ± 98†	151.9 ± 8.1†	6.45 ± 1.00†
Aroclor 1254 + SIM	829 ± 136	112.5 ± 15.3‡	7.72 ± 1.36

* Drug treatment as described in legend to Table 1. The livers were taken on day 11 of the experiment. The values represent means ± SD of three rats per group.
† Significant differences from control ($P < 0.05$).
‡ Significant differences from Aroclor 1254 ($P < 0.05$).

SIM, and by a factor of 5.8 for SIM and 5.1 for SIM + metabolites after ten administrations. Concentrations of SIM measured 5 hr after the last administrations were, however, similar in rats treated with SIM and in rats treated with Aroclor 1254 + SIM. In addition, the concentration ratio SIM + metabolites/SIM clearly showed that immunoreactive metabolites were more abundant after five than after ten administrations of SIM. This observation can be explained by a reduction of SIM

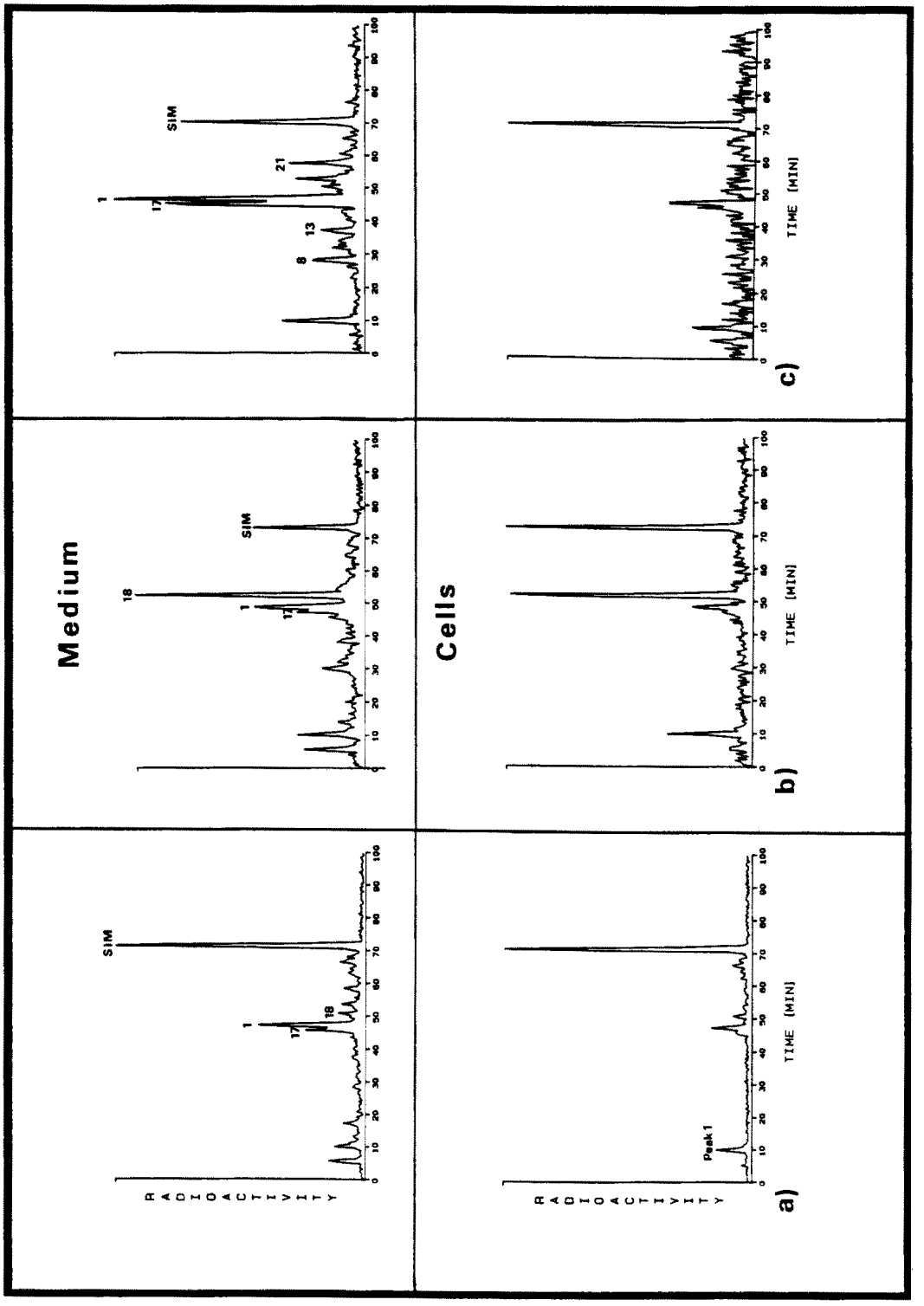

Fig. 1. *In vitro* metabolite patterns of SIM. Metabolite patterns in medium (upper panels) and in cells (lower panels) by HPLC (see Materials and Methods) after 17 hr incubation of 1 μM SIM in cultures of rat hepatocytes obtained from (a) control rats, (b) Aroclor 1254-induced rats, (c) dexamethasone-induced rats.

metabolism, and is confirmed by the fact that SIM concentrations were higher after ten doses than after five.

Liver enzymes. Treatment of the rats with Aroclor 1254 caused a 3.7-fold increase in hepatic cytochrome P-450 concentration and a significant elevation of ethoxycoumarin-*O*-deethylase and cytochrome P-450 reductase activities by factors of 13.4 and 2.1, respectively, as compared to control rats (Table 3). Treatment with SIM did not change any of these hepatic parameters of biotransformation versus control. During concomitant treatment with Aroclor 1254, SIM inhibited the induction of cytochrome P-450 reductase activity by 26% in comparison to Aroclor 1254-induced rats.

In vitro *experiments*

Metabolism of SIM. It was previously shown that the uptake of [^{3}H]SIM into hepatocyte cultures from untreated rats occurred rapidly and in a concentration-dependent manner, and that a steady-state was reached after 1 hr [22]. Figure 1 shows the metabolite patterns both in the medium and hepatocytes after 17 hr incubation of hepatocyte cultures obtained from control and Aroclor 1254- or dexamethasone-pretreated rats, in the presence of 1 μM [^{3}H]SIM. The primary cultures of rat hepatocytes were, therefore, able to metabolize SIM and to secrete the metabolites into the medium. In control cultures, the major metabolites observed in the media were the three monohydroxlated metabolites M1, M17 and M18 [17, 18]. These metabolites were also found in the cells. Treatment of the rats with Aroclor 1254 greatly enhanced the formation of M18 found in the medium and in the cells. The concentrations of the two other metabolites however, remained identical to those in the control cells. Pretreatment of the rats with dexamethasone led to an increased production of M1 and M17, but not M18. Three other metabolites which were not found in control hepatocyte cultures, namely M21, M13, and M8, appeared in the medium (Fig. 1). Quantitatively, 51% (1 μM extracellular SIM) and 27% (10 μM extracellular SIM) of the total SIM previously added

Table 4. Effects of *in vivo* pretreatment with Aroclor 1254 or dexamethasone on the rate of SIM metabolism *in vitro**

Pretreatment	SIM (μM)	Non-metabolized SIM (% of respective total)†		
		Medium	Cells	Total‡
None	1	42.8	66.4	48.7
	10	68.7	83.0	72.7
Dexamethasone	1	20.7	28.6	22.3
	10	71.1	74.7	71.8
Aroclor 1254	1	12.5	39.3	18.6
	10	56.3	87.0	65.3

* The rats received two intraperitoneal injections of either Aroclor 1254 or dexamethasone (100 mg/kg), six and three days prior to the hepatocyte isolation. [^{3}H]SIM was added to the hepatocyte cultures at the indicated concentrations and incubated for 17 hr. The fraction of non-metabolized SIM was determined by HPLC using a radiodetection method. The data represent means of duplicate determinations from one batch of hepatocytes.

‡ Per cent of total radioactivity in medium + cells attributable to SIM.

† Per cent of total radioactivity in medium + cells attributable to SIM.

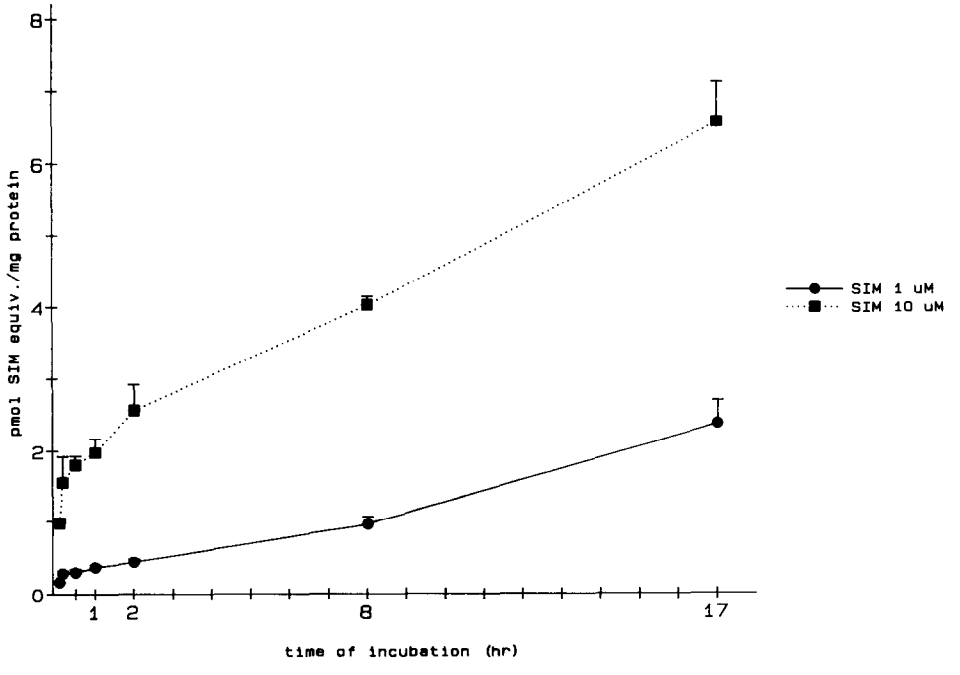

Fig. 2. [^{3}H]SIM or [^{3}H]SIM metabolites covalently bound to hepatic macromolecules as a function of culture time. The values represent means ± SD of three plates per time point from one batch of hepatocytes. For details, see Materials and Methods.

Table 5. Intracellular concentrations and covalent binding of [^{3}H]SIM or [^{3}H]SIM metabolites to hepatic macromolecules in cultured hepatocytes obtained from control and Aroclor 1254- or dexamethasone-induced rats*

Pretreatment	Incubation (hr)	SIM (μM)	Cell-associated SIM + metabolites (pmol equiv./mg protein)	Covalent binding (pmol equiv./mg protein)	Binding index† (%)
None	2	1	475 ± 26	0.44 ± 0.04	0.09
	17	1	444 ± 30	2.36 ± 0.36	0.53
	2	10	4558 ± 353	2.55 ± 0.36	0.06
	17	10	5497 ± 548	6.54 ± 0.55	0.12
Dexamethasone	2	1	226 ± 25	1.79 ± 0.49	0.79
	17	1	179 ± 20	9.22 ± 2.62	5.15
	2	10	2974 ± 303	20.34 ± 4.78	0.68
	17	10	2124 ± 374	67.86 ± 8.24	3.20
Aroclor 1254	17	1	549 ± 31	4.14 ± 0.05	0.82

* *In vivo* drug pretreatment as described in legend to Table 4. [^{3}H]SIM was added to the hepatocyte cultures at the indicated concentrations and incubated for 2 or 17 hr. Cell-associated radioactivity and covalent binding of [^{3}H]SIM was determined as described in Materials and Methods. The data represent means ± SD of triplicate determinations from one batch of hepatocytes.

† Fraction of covalently bound radioactivity per total cell-associated SIM + SIM metabolites.

to the cultures were biotransformed in control cultures during 17 hr (Table 4), whereas after 2 hr incubation, only a minor portion of SIM was metabolized, even in "induced" cultures (data not shown). Dexamethasone and Aroclor 1254 pretreatment increased the capacity for SIM biotransformation. At 1 μM SIM, both inducers increased the rate of metabolism by a factor of 2 to 3 (Table 4). In contrast, at 10 μM SIM, the induction with Aroclor 1254 had only a minor effect on the rate of SIM metabolism, whereas dexamethasone-induction had no effect.

Covalent binding. Covalent binding of SIM and/or SIM metabolites to cellular constituents occurred rapidly during the first hour of exposure. This process continued thereafter in a linear time-proportional fashion up to the end of incubation (Fig. 2). After 17 hr incubation using 1 or 10 μM SIM, the covalent binding amounted to 2364 ± 322 or 6543 ± 555 fmol of SIM associated radioactivity per mg hepatic protein, respectively. These values represented 0.53 or 0.12% of the total intracellular amount of SIM. Pretreatment of the rats with dexamethasone increased the covalent binding of SIM to hepatocyte proteins by a factor of 4 using 1 μM extracellular SIM concentration, and by a factor of 8–10 using 10 μM SIM as summarized in Table 5. These values were not proportional to the intracellular steady-state concentrations of SIM and metabolites. Pretreatment with dexamethasone, was associated with a two-fold decrease of the intracellular SIM concentration as compared to untreated rats (Table 5). Aroclor 1254 pretreatment, on the other hand, did not alter intracellular steady-state SIM concentrations and was less potent than dexamethasone in augmenting the covalent binding of SIM and/or SIM metabolites to cell proteins.

Protein biosynthesis. As previously described [32], the hepatocytes are able to synthesize proteins from [^{3}H]-labeled amino acids added to the culture medium. Furthermore, the measurement of SIM-induced inhibition of this synthesis appeared to be a sensitive cytotoxicity test [22]. Inhibition of total protein biosynthesis was, therefore, chosen as an *in vitro* end-point for SIM toxicity assessment. In accordance with our previous findings, SIM inhibited protein synthesis in a concentration-dependent manner in untreated cells, and in Aroclor 1254-induced hepatocytes (40% inhibition at 10 μM extracellular SIM after 17 hr incubation). Similarly, in cells obtained from dexamethasone-treated rats, the slope of the inhibition curve was identical to that of the control cultures, although a slight increase in protein synthesis was observed using low SIM concentrations. It can therefore be concluded that *in vitro* inhibition of protein biosynthesis induced by SIM was identical in Aroclor 1254- or dexamethasone induced rats to that in control rats.

DISCUSSION

The mechanism of SIM-induced hepatotoxicity has not yet been completely elucidated. However, recent evidence indicates that the clinical manifestations of SIM hepatotoxicity are associated with predictable, dose-dependent impairment of specific cellular functions. First, bile salt transport across hepatocytes into bile is inhibited by SIM, resulting in cholestasis [11, 12]. Our results are in agreement with these findings (Table 1). Second, it has been demonstrated that SIM inhibits protein synthesis *in vitro* and *in vivo*, which can explain the decrease of total plasma proteins measured in rats chronically treated with SIM [13, 33, 34]. The results of the present study corroborate these well known rodent-specific manifestations of SIM hepatotoxicity (Table 1, Fig. 3). Commensurate with the absence of overt histopathological alterations, plasma activities of ALT were decreased during treatment with high doses of SIM (Table 1). Similarly, in hepatocyte cultures, LDH leakage, a marker for the integrity of the plasma membrane, was not increased at the toxic concentrations used in our experiments [22]. Therefore, this endpoint was not included as a toxicity measure for our *in vitro* experiments with SIM.

SIM is extensively metabolized by the hepatic cytochrome P-450-dependent monooxygenases [17–19], and mainly excreted via the biliary system [26].

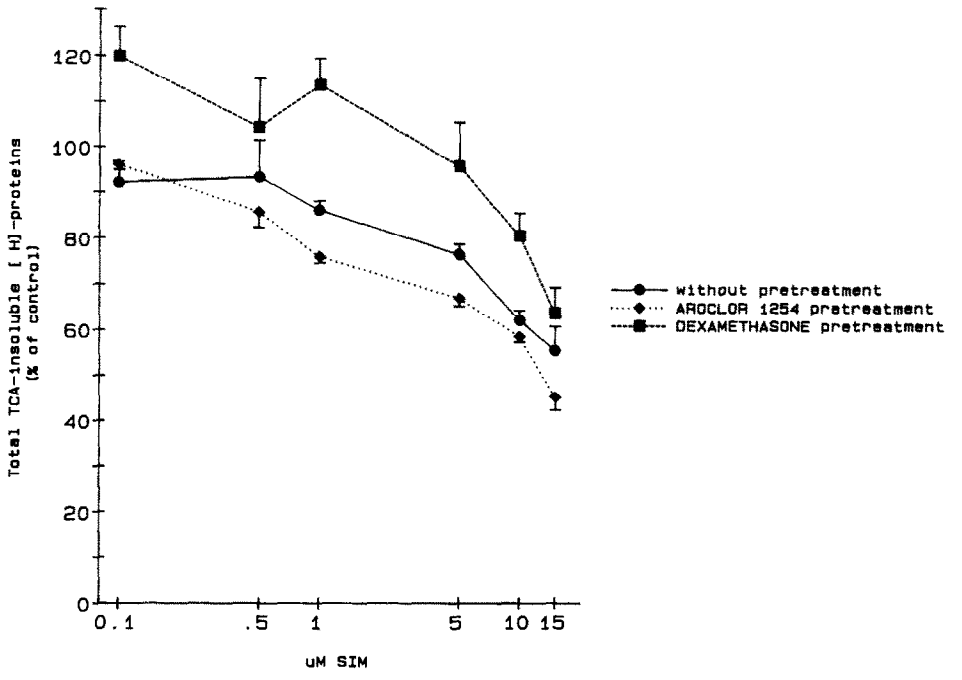

Fig. 3. *In vitro* inhibition of total protein biosynthesis in the presence of various concentrations of SIM in cultures of rat hepatocytes obtained from control and Aroclor 1254- or dexamethasone-induced rats. The values represent the incorporation of [^{3}H]amino acids into TCA-insoluble proteins after 17 hr incubation, and are the means ± SD of three plates from one batch of hepatocytes. For details, see Materials and Methods.

In order to study the role of increased SIM metabolism and plasma disappearance on the degree of hepatotoxicity, the biotransformation capacity was induced with either Aroclor 1254 or dexamethasone. The former substance induces a broad spectrum of cytochrome P-450 isozymes including PB-like and 3-MC-like isozymes [35, 36], whereas dexamethasone induces the cytochrome P-450 III gene family which is responsible for the formation of the primary SIM metabolites M1, M17, and M21 in the rabbit [37, 38] and in man [19]. Both inducers markedly increased SIM biotransformation (Tables 2 and 4, Fig. 1), which correlated with increased contents and activities of cytochrome P-450 (Table 3). However, none of these pretreatments with metabolic inducers altered the quantitative response to the toxic insult (elevated bilirubin and bile salt concentrations, reduced total plasma protein, inhibition of protein biosynthesis) induced by SIM *in vivo* and *in vitro* (Table 1, Fig. 3). Earlier preliminary data indicated that only LDH leakage induced by excessively high concentrations of SIM was markedly decreased in Aroclor 1254-pretreated hepatocytes [39].

The monitoring of SIM plasma concentrations and its metabolites using HPLC and RIA (Table 2) and resulting calculated kinetic data are in accordance with the findings of Wagner *et al.* [26], who reported that 24 hr after a single oral administration of 10 mg/kg [^{3}H]SIM, 60% of total radioactivity was present as unchanged SIM and that multiple administration of SIM resulted in its accumulation. Our results confirm and extend these findings. The lack of effect of Aroclor 1254 on SIM accumulation in plasma can be correlated with a three-fold increased rate of SIM elimination. Furthermore, the $T_{\frac{1}{2}}$ of elimination between 5 and 24 hr was higher when measured by RIA than determined by HPLC, indicating a higher rate of elimination for metabolites than for the parent compound. Qualitatively, the identification of the individual metabolites, generated *in vitro* using [^{3}H]SIM (Fig. 1), revealed two major metabolites (M1 and M17) which had also been identified *in vivo* [18, 26]. Recent physico-chemical and toxicological studies indicated that the metabolite M17 was more polar and less toxic than SIM itself [21, 22]. The toxicity of other metabolites such as, e.g., M18, which appeared in large amounts in Aroclor 1254-induced hepatocyte cultures, has not been studied so far. However, in the course of metabolic activation, some unknown metabolites reacted with cellular targets, as shown by covalent binding of SIM to hepatic macromolecules and its marked increase after dexamethasone induction. This confirms and extends the findings of Nagelkerke *et al.* [40], who demonstrated that small fractions of SIM were covalently bound to S9 rat liver preparations, to macromolecules of isolated rat hepatocytes, and to the rat liver *in vivo*. In contrast, the absence of significant binding of [^{3}H]SIM and/or its metabolites to cellular macromolecules of the mouse liver has been reported by others [41]. However, no quantitative or qualitative data on the metabolic conversion of SIM in mice were provided in that study. The specific rat liver proteins to which the reactive SIM metabolites were covalently bound have not been characterized. As not all covalent binding of reactive intermediates to cellular proteins need to elicit toxicity, the toxicological significance of the binding of SIM to macro-

molecules of rat hepatocytes still remains unknown. The absence of modulating effects of an induction of hepatic drug metabolism on SIM-induced hepatotoxicity is in contrast to the amelioration of nephrotoxicity observed after Aroclor 1254-induction [23–25]. Besides major differences between the mechanisms of SIM-induced nephrotoxicity and hepatotoxicity, other factors, such as high portal SIM concentrations or the liver being the principal site of SIM biotransformation and excretion, might contribute to this discrepancy. Thus, hepatocellular SIM concentrations rather than SIM plasma disappearance rates seem to determine the extent of hepatic dysfunction in the rat.

Acknowledgements—The authors gratefully acknowledge the expert technical assistance of Ms M. B. Homberger, Ms M. Tschopp and Mr S. Wüthrich. We would also like to thank Prof. Ch. Schlatter for his continuous support and critical discussions.

REFERENCES

1. Myers BD, Ross J, Newton L, Luetscher J and Perlroth M, Cyclosporine-associated chronic nephropathy. *N Engl J Med* **331**: 699–705, 1984.
2. Ryffel B and Mihatsch MJ, Cyclosporine nephrotoxicity. *Toxicol Pathol* **14**: 73–82, 1986.
3. Cohen DJ, Loertscher R, Rubin MF, Tilney NL, Carpenter CB and Strom TB, Cyclosporine: a new immunosuppressive agent for organ transplantation. *Ann Int Med* **101**: 667–682, 1984.
4. Atkinson K, Biggs J, Dodds A and Concannon A, Cyclosporine-associated hepatotoxicity after allogeneic marrow transplantation in man: differentiation from other causes of posttransplant liver disease. In: *Cyclosporine: Biological Activity and Clinical Applications* (Ed. Kahan BD), pp. 545–551. Grune & Stratton, Orlando, 1984.
5. Klintmalm GBG, Iwatsuki S and Starzl TE, Cyclosporin A hepatotoxicity in 66 renal allograft recipients. *Transplant* **32**: 488–489, 1981.
6. Hillebrand G, Castro LA, Habersetzer R, Stoffner D, Gokel JM, Gurland HJ and Land W, Chronic cyclosporine hepatotoxicity after renal transplantation. *Transplant Proc* **18**: 1020–1022, 1986.
7. Lorber MI, Van Buren CT, Flechner SM, Williams C and Kahan BD, Hepatobiliary and pancreatic complications of cyclosporine therapy in 466 renal transplant recipients. *Transplant* **43**: 35–40, 1987.
8. Rodger RSC, Turney JH, Haines T, Michael J, Adu D and McMaster P, Cyclosporine and liver function in renal allograft recipients. In: *Cyclosporine: Biological Activity and Clinical Applications* (Ed. Kahan BD), pp. 538–540, Grune & Stratton, Orlando, 1984.
9. Laupacis A, Keown PA, Ulan RA, Sinclair NR and Stiller CR, Hyperbilirubinaemia and cyclosporin A levels. *Lancet* **19**: 1426–1427, 1981.
10. Loertscher R, Wenk M, Harder F, Brunner F, Follath F and Thiel G, Hyperbilirubinaemia and cyclosporin A levels in renal transplant patients. *Lancet* **19**: 635–636, 1981.
11. Schade RR, Guglielmi A, Van Thiel DH, Thompson ME, Warty V, Griffith B, Sanghvi A, Bahnson H and Hardesty R, Cholestasis in heart transplant recipients treated with cyclosporine. *Transplant Proc* **15**: 2757–2760, 1983.
12. Stone BG, Udani M, Sanghvi A, Warty V, Plocki K, Bedetti CD and Van Thiel DH, Cyclosporin A-induced cholestasis. The mechanism in a rat model. *Gastroenterology* **93**: 344–351, 1987.
13. Buss WC, Stepanek J and Bennett WM, Proposed mechanism of cyclosporine toxicity: inhibition of protein synthesis. *Transplant Proc* **20**: 863–867, 1988.
14. Stacey NH and Kotecka B, Inhibition of taurocholate and ouabain transport in isolated rat hepatocytes by Cyclosporin A. *Gastroenterology* **95**: 780–786, 1988.
15. Kukongviriyapan V and Stacey N, Inhibition of taurocholate transport by cyclosporin A in cultured rat hepatocytes. *J Pharmacol Exp Ther* **257**: 685–689, 1988.
16. Boelsterli UA, Bouis P and Donatsch P, Effect of cyclosporin A on bile acid uptake, conjugation and release in cultured rat hepatocytes. In: *Liver Cells and Drugs*. Collq. INSERM (Ed. Guillouzo A), pp. 335–341. John Libbey Eurotext, Ltd, London, 1988.
17. Maurer G, Metabolism of cyclosporine. *Transplant Proc* **17**: 19–26, 1985.
18. Maurer G and Lemaire M, Biotransformation and distribution in blood of cyclosporine and its metabolites. *Transplant Proc* **18** (suppl.5): 25–34, 1986.
19. Kronbach T, Fischer V and Meyer UA, Cyclosporine metabolism in human liver: identification of a cytochrome P-450III gene family as the major cyclosporine metabolizing enzyme explains interactions with other drugs. *Clin Pharmacol Ther* **43**: 630–635, 1988.
20. Burckart GJ, Starzl TE, Venkataramanan R, Hashim H, Wong L, Wang P, Makowka L, Zeevi A, Ptachcinski RJ, Knapp JE, Iwatsuki S, Esquivel C, Sanghvi A and Van Thiel DH, Excretion of cyclosporine and its metabolites in human bile. In: *Cyclosporine: Biological Activity and Clinical Applications* (Ed. Kahan BD), pp. 46–49. Grune & Stratton, Orlando, 1984.
21. Ryffel B, Hiestand P, Foxwell B, Donatsch P, Boelsterli HJ, Maurer G and Mihatsch MJ, Nephrotoxic and immunosuppressive potentials of cyclosporine metabolites in rats. In: *Cyclosporine: Biological Activity and Clinical Applications* (Ed. Kahan BD), pp. 41–45. Grune & Stratton, Orlando, 1984.
22. Boelsterli UA, Bouis P, Brouillard JF and Donatsch P, *In vitro* toxicity assessment of cyclosporin A and its analogs in a primary rat hepatocyte culture model. *Toxicol Appl Pharmacol* **96**: 212–221, 1988.
23. Cunningham C, Burke MD, Wheatley DN, Thomson AW, Simpson JG and Whiting PH, Amelioration of cyclosporin-induced nephrotoxicity in rats by induction of hepatic drug metabolism. *Biochem Pharmacol* **34**: 573–578, 1985.
24. Duncan JI, Heys SD, Thomson AW, Simpson JG and Whiting PH, Influence of the hepatic drug-metabolizing enzyme-inducer phenobarbitone on cyclosporine nethrotoxicity and hepatotoxicity in renal-allografted rats. *Transplant* **45**: 693–697, 1988.
25. Whiting PH, Thomson AW and Simpson JG, Cyclosporine: toxicity, metabolism, and drug interactions—implication from animal studies. *Transplant Proc* **17**: 134–144, 1985.
26. Wagner O, Schreier E, Heitz F and Maurer G, Tissue distribution, disposition, and metabolism of cyclosporine in rats. *Drug Metab Dispos* **15**: 377–383, 1987.
27. Donatsch P, Abisch E and Homberger M, A radioimmunoassay to measure cyclosporin A in plasma and serum samples. *J Immunol* **2**: 19–32, 1981.
28. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1976.
29. Greenlee WF and Poland A, An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methyl-cholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Pharmacol Exp Ther* **205**: 596–605, 1978.
30. Falzon M, Milton AS and Burke MD, Are the decreases in hepatic cytochrome P-450 and other drug-metabolising enzymes caused by indomethacin *in vivo*

mediated by intestinal bacterial endotoxins? *Biochem Pharmacol* **33**: 1285–1292, 1984.
31. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analyt Biochem* **72**: 248–254, 1976.
32. Boelsterli UA, Bouis P and Donatsch P, Relative cytotoxicity of psychotropic drugs in cultured rat hepatocytes. *Cell Biol Toxicol* **3**: 231–250, 1987.
33. Blair JT, Thompson AW, Whiting PH, Davidson RJL and Simpson JG, Toxicity of the immune suppressant cyclosporin A in the rat. *J Path* **138**: 163–178, 1982.
34. Bäckman L, Appelkvist EL, Ringden O and Dallner G, Effects of cyclosporin A on hepatic protein synthesis. *Transplant Proc* **20** (Suppl. 3): 853–858, 1988.
35. Ryan DE, Thomas PE, Reik LM and Levin W, Purification, characterization and regulation of five rat hepatic microsomal cytochrome P-450 isozymes. *Xenobiotica* **12**: 727–744, 1982.
36. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or beta-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
37. Fabre G, Bertault-Peres P, Fabre I, Maurel P, Just S and Cano JP, Metabolism of cyclosporin A. I. Study in freshly isolated rabbit hepatocytes. *Drug Metab Dispos* **15**: 384–390, 1987.
38. Bertault-Peres P, Bonfils C, Fabre G, Just S, Cano JP and Maurel P, Metabolism of cyclosporin A. II. Implication of the macrolide antibiotic inducible cytochrome P-450 3c from rabbit liver microsomes. *Drug Metab Dispos* **15**: 391–398, 1987.
39. Bouis P, Fischer V and Boelsterli UA, Modulation of cyclosporin A toxicity in cultured rat hepatocytes by induction of the biotransformation capacity. *Experientia* **44**: A53, 1988.
40. Nagelkerke JF, Tijdens RB, Schwartz EP, Winters MFG, Leendert CP and Mulder GJ, The covalent binding of cyclosporin A to rat liver macromolecules *in vivo* and *in vitro*: the role of the cytochrome P-450. *Toxicology* **47**: 277–284, 1987.
41. Bäckman L, Brandt I, Appelkvist EL and Dallner G, Tissue and subcellular localizations of [^{3}H]-cyclosporine A in mice. *Pharmacol Toxicol* **62**: 110–117, 1988.