CYCLOSPORINE PHARMACOLOGY IN ANIMALS

Predominance of Native Cyclosporine Over Metabolites in Rat Blood and Tissue

M.A. Pell, T.G. Rosano, K.L. Brayman, B.M. Freed, L.M. Shaw, and N. Lempert

THE RAT MODEL has been used extensively in attempts to understand cyclosporine (CsA)-induced nephrotoxicity, which remains the major impediment to the safe clinical usage of CsA. Nephrotoxicity in the rat model has been characterized by histologic changes in the renal tubules, alterations in tubular function and a more limited impairment in glomerular filtration rate (GFR), as measured by creatinine clearance.[1-5] The dose of CsA required to induce these nephrotoxic effects in rats, however, far exceeds the dose associated with nephrotoxicity in humans. In humans,[6,7] for example, CsA at doses of 5 to 20 mg/kg/d was accompanied by nephrotoxicity, while in Wistar rats[8] a decrease in serum creatinine or creatinine clearance was not observed after 30 days of CsA treatment at a dose of 20 mg/kg/d. Appreciable nephrotoxicity in rats was observed in other studies when CsA doses of 45 to 100 mg/kg/d were used.[1,3]

Modulation of nephrotoxicity in rats has also been achieved by induction of the P-450 enzyme system, which converts CsA to its metabolites.[9,10] This finding suggests that CsA metabolism plays a role in the nephrotoxicity, and it is possible that resistance to nephrotoxicity in the rat may be related to differences in metabolism between rats and humans. CsA metabolism in rats was studied by Maurer et al after administration of tritiated CsA and showed that blood and tissue contain predominantly CsA, with metabolite 1 (M1) being the major metabolite.[11] With the development of high-performance liquid chromatography (HPLC) methods for the specific measurement of CsA and its primary metabolites, the levels of these compounds have been quantitated in blood and renal tissue from human renal allograft recipients. Blood and tissue from humans treated with CsA contain predominantly metabolite 17 (M17) and lesser amounts of CsA and M1.[12,13] Recent studies in humans have also shown the accumulation of additional polar metabolites during an episode of nephrotoxicity.[14] The purpose of the present study was to evaluate CsA and metabolite levels in rat blood and tissue by HPLC and to compare the pattern of metabolism in rats with metabolite patterns reported for humans.

MATERIALS AND METHODS

Experiments were conducted with adult male Wistar-Furth rats (200 to 250 g), which were bred and main-

From the Departments of Clinical Chemistry and Biochemistry, Albany Medical Center, NY; and the Departments of Surgery, Pathology, and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia.

Address reprint requests to Dr Thomas G. Rosano, Clinical Laboratory Services, Albany Medical Center Hospital, Albany, NY 12208.

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tained at the University of Pennsylvania or were obtained from Harland Sprague Dawley Farm (Indianapolis, IN). Treatment was initiated after a minimum of 1 week acclimatization. For oral CsA treatment, CsA (Sandimmune oral solution 100 mg/mL, Sandoz, Basel, Switzerland) was diluted with olive oil (Filippo Berio Co, Lucca, Italy) to a final concentration of 10 mg/mL. Conscious rats received a CsA dose of 15 mg/kg/d of body weight by gastric intubation. For subcutaneous (SC) CsA treatment, CsA (Sandimmune IV solution, Sandoz) was diluted under sterile conditions with physiologic saline to a final concentration of 10 mg/mL. Conscious rats received 15 mg/kg/d by SC injection under the dorsal skinfold behind the neck. Before initiating CsA treatment, and 24 hours after the last dose, blood was collected from clean cut tail tips of rats anesthetized with sodium pentobarbital (35 to 40 mg/kg, administered intraperitoneally). Aliquots of whole blood were stored at −20°C until they could be analyzed for cyclosporines. Immediately after final blood collection, kidney and liver tissues were excised and stored in 0.9% NaCl at −20°C. Tissue were then thawed, weighed, and a 500- to 700-mg piece was finely minced and homogenized in five volumes of a 0.9% NaCl solution, using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The volume of the homogenate was adjusted with 0.9% NaCl to give a final tissue concentration of 100 mg/mL.

Additional experiments were performed with 15-week-old spontaneously hypertensive rats obtained from Taconic Farms (Germantown, NY). After acclimitization, these rats were treated with CsA (75 mg/kg/d) for seven or 14 days by the oral dosing protocol described above with the exception that the Sandimmune was administered undiluted. Whole blood was also collected before and after the dosing regimen as described above. Serum creatinine analysis was performed (SMAC autoanalyzer, Tarrytown, NY) on serum samples collected prior to and at the end of the CsA treatment protocol.

Specific quantitation of CsA, M1, M17, and M21 concentrations was performed as previously described.[13] Briefly, 0.5 to 1 mL of whole blood or tissue homogenates were ether-extracted under alkaline then acid conditions. After ether evaporation, CsA and the metabolites were dissolved in 300 μL of mobile phase (43% acetonitrile/57% H_2O) and subjected to a final ether extraction, heptane wash, and evaporation. The dried extract was reconstituted with 200 μL of mobile phase and a 50 μL sample was chromatographed on a cyanpropyl column (4.6 mm × 25 cm; Dupont Instruments, Wilmington, DE) maintained at 65°C with a flow rate of 0.5 mL/min. Peaks were detected at 215 nm, and the assay was standardized with blood supplemented with CsA, M1, M17 and M21 (generous gift of Dr. G. Maurer, Biopharmaceutical Department, Sandoz Ltd, Basel, Switzerland). Dihydrocyclosporine C (CsC) was used as an internal standard and the peak height ratio of calculation was used for quantitation. In the HPLC analysis of CsA and metabolites in rat blood and tissue from nontreated animals, peaks coeluting with CsA and the primary metabolites were not observed. All blood and tissue samples were analyzed in duplicate.

HPLC fractionation studies were also performed to determine whether other metabolites of CsA were present. Fractions (1 mL) from the HPLC column effluent were collected during the analysis of CsA and primary metabolites, and replicate aliquots (100 μL) were dried for subsequent analysis by an RIA method (RIA-NS) from Sandoz Ltd. The RIA assay used a nonspecific monoclonal antibody, developed by Quesniaux et al, and which reacts with CsA and its metabolites.[15,16] RIA reagents were obtained from Sandoz, Ltd and the only modification to the manufacturer's protocol was the method of sample extraction. In our studies, HPLC fraction aliquots were redissolved in 1 mL of methanol, a 50-μL sample was added to the reaction mixture, and the subsequent competitive binding analysis was carried out according to the manufacturer's procedure. The binding depression was compared to CsA standards and the reactivity of fractions was expressed in CsA-equivalent nanograms per fraction.

RESULTS

Figure 1 shows the CsA, M1, and M17 concentrations in trough blood, kidney, and liver tissue homogenates of Wistar-Furth rats treated with CsA (15 mg/kg/d) for four to 32 days. In blood, the parent compound was the predominant cyclosporine, with CsA concentrations five to six fold greater than the combined concentrations of M1 and M17. CsA was also the major component in kidney and liver tissue, representing four times the amount of primary metabolites. M1 was the predominant metabolite throughout the treatment period, followed by lesser amounts of M17. M21 was detected in only a few blood and tissue samples. Analysis of variance for blood and tissue data showed no statistically significant change in the mean CsA, M1, and M17 concentrations from days 4 to 32, indicating that trough blood, kidney and liver tissue levels of CsA and primary metabolites reached steady states by the fourth day of treatment.

We also examined the relationship between the blood and tissue content of CsA and metabolites. The kidney and liver tissue concentrations of CsA and the metabolites were

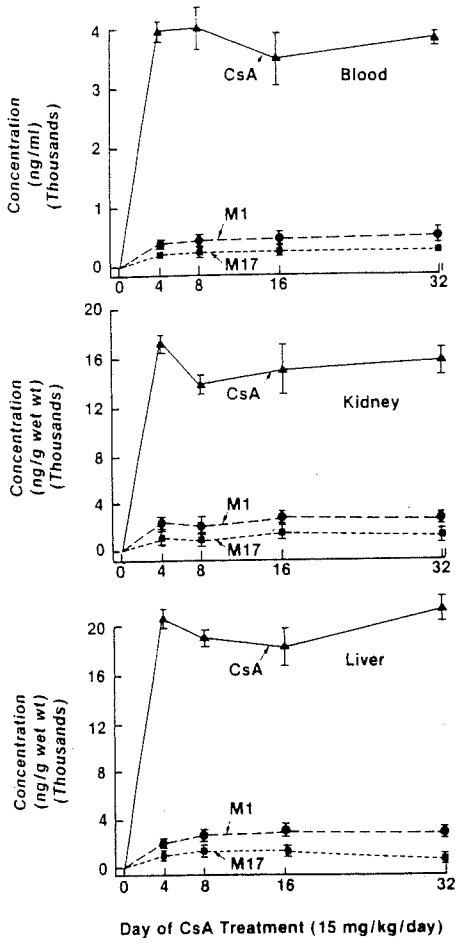

Fig 1. CsA and metabolite concentrations in blood, kidney and liver tissue from Wistar-Furth rats treated SC with 15 mg/kg/d. Male rats weighing 200 to 300 g were treated SC with CsA (15 mg/kg/d) and killed 24 hours after the final dose (trough) on days 4, 8, 16, or 32. A control group of animals was not treated with CsA and was killed on day 0. Concentration data for each of the days represent the mean and standard deviation for three animals. Analysis of variance was performed and showed no statistically significant difference between the mean concentrations of CsA or metabolites on days 4, 8, 16, and 32. M21 was not detected in any of the samples.

40 to 50 times greater than in blood on a per gram basis. For example, over the treatment period the mean M1 and M17 concentrations in kidney (2.37 μg/g and 1.04 μg/g, respectively) were similar to concentrations in liver tissue (2.53 μg/g and 1.07 μg/g) but were much greater than the corresponding concentrations in blood (0.45 μg/mL and 0.25 μg/mL). For the parent drug, the mean concentration in liver (19.7 μg/g wet weight) was not significantly greater than in kidney tissue (15.4 μg/g wet weight), but was again much greater than in blood (3.85 μg/mL). Although absolute concentrations of cyclosporines were much higher in tissues, the ratios of metabolite to CsA concentrations were similar in blood and tissue. For example, the M1/CsA ratio in blood was 0.12, compared to ratios of 0.16 and 0.13 for kidney and liver, respectively. The ratio of M17/CsA in blood (0.07), kidney (0.07) and liver (0.06) also showed similar relative amounts of metabolites and parent drug in blood and tissue. These findings indicate that the concentrations of CsA and metabolites in blood reflect the relative proportions in both kidney and liver tissue and suggest that an equilibrium process exists between blood and tissue.

The effect of route of administration on trough blood concentrations of CsA and metabolite was also determined in additional experiments with the Wistar-Furth rats. As can be seen in Table 1, primary metabolite concentrations were similar in animals treated with oral or SC CsA, while the parent drug concentration in the subcutaneous group was

Table 1. Effect of Route of Administration on CsA Metabolism in Wistar-Furth Rats Treated With CsA (15 mg/kg/d) for Eight Days

	Through Blood Concentration (ng/mL)	
Compound	Oral	Sc
M17	196 ± 53	250 ± 52
M1	359 ± 106	461 ± 43
CsA	1,677 ± 23	4,038 ± 806*

NOTE. Male Wistar-Furth rats, 200 to 300 g, were treated orally (N = 4) with CsA (Sandimmune oral solution diluted with olive oil) by gavage and SC (N = 5) by injection (Sandimmune IV solution diluted with sterile physiologic saline). M21 was not detected in any of the blood samples analyzed. In all samples the M21 concentration was less than the detection limit (20 ng/mL) of the HPLC method. Results are mean ± SD.

*Significant difference ($P < .01$) in the mean concentration when compared to orally treated rats.

significantly greater than in the orally treated group. These analyses showed that with either SC or oral administration the pattern of CsA and primary metabolites was similar (CsA ≫ M1 > M17) but that proportionately smaller amounts of CsA were present in blood after oral treatment.

In order to determine whether CsA metabolites other than M1 and M17 were present in blood from CsA-treated rats, HPLC fractions of trough blood were collected and analyzed by a monoclonal RIA method (RIA-NS) that detects CsA and metabolites. Figure 2 shows a representative fractionation study of blood from a Wistar-Furth rat treated with CsA (15 mg/kg/d). The upper panel shows ultraviolet monitoring at 215 nm, which measured M17 (240 ng/mL), M1 (478 ng/mL), and CsA (1,872 ng/mL). Further analysis of HPLC fractions by the monoclonal RIA method (lower panel) showed greater than 98% of the RIA-NS reactivity of blood in fractions containing CsA, M1, and M17. Reactivity in fractions 17 and 18 was due to the internal standard (CsC), which was added as during the analytic preparation of the sample. Additional metabolites were therefore not detected in the blood of these rat indicating that CsA, M1, and M17 were the major cyclosporines under this treatment protocol.

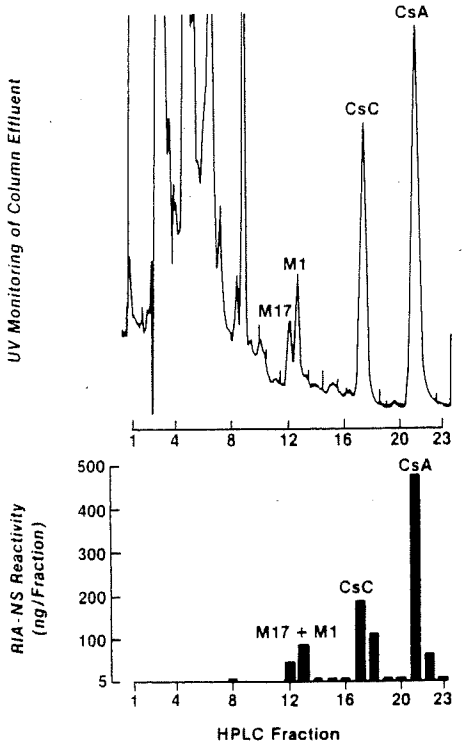

Fig 2. HPLC chromatogram and fractionation study of trough blood from a Wistar-Furth rat treated orally with CsA (15 mg/kg/d) for eight days. The upper panel shows the column effluent monitoring by ultraviolet detection (215 nm). Concentrations of CsA (1,651 ng/mL), M1 (357 ng/mL), and M17 (212 ng/mL) were determined by HPLC. The corresponding RIA-NS reactivity of the fractions is displayed in the lower panel.

Table 2. Trough Blood Concentrations of CsA and its Primary Metabolites in Spontaneously Hypertensive Rats

Compound	Trough Blood Concentration (ng/mL) Day 7*	Day 14†
M17	1,076 ± 128	992 ± 182
M1	2,476 ± 443	1,740 ± 411
M21	80 ± 34	133 ± 98
CsA	13,051 ± 2,127	14,276 ± 853

NOTE. Male Spontaneously hypertensive rats 275 to 300 g, were treated orally with CsA (75 mg/kg/d, Sandimmune oral solution diluted with olive oil) by gavage for 7 or 14 days. Results are mean ± SD.

*Four rats treated.

†Three rats treated.

The pattern of CsA metabolites was also studied in spontaneously hypertensive rats, which have been used widely in the study of CsA-induced nephrotoxicity. After oral administration of a high dose of CsA (75 mg/kg/d) to groups of animals treated for either seven or 14 days, the trough blood concentrations of CsA and metabolites were determined (Table 2). The higher daily dose resulted in significantly higher concentrations of CsA and primary metabolites when compared to the Wistar-Furth rat treated with 15 mg/kg/d. The relative order of concentrations (CsA ≫ M1 > M17), as well as the ratios of M1/CsA and M17/CsA concentrations, were, however, similar in both rat strains despite the differences in dose. The HPLC fractionation studies were also performed on trough blood samples from rats treated seven

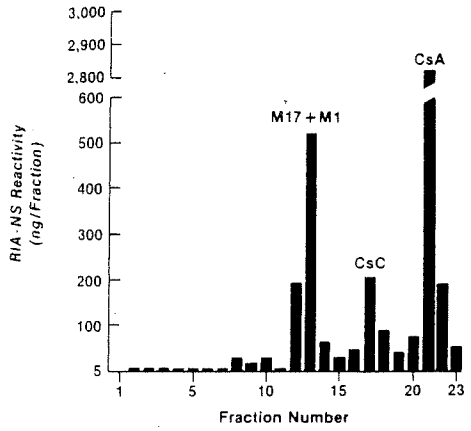

Fig 3. RIA-NS reactivity of HPLC fractions of blood obtained from a spontaneously hypertensive rat treated orally with CsA (75 mg/kg/d) for seven days. The concentrations of CsA (11,468 ng/mL), M1 (2,658 ng/mL), M17 (1,135 ng/mL), and M21 (73 ng/mL) were determined by HPLC.

and 14 days, and a representative study is displayed in Fig 3. Blood samples from both the seven- and 14-day treatment groups showed that greater than 98% of the endogenous RIA-NS reactivity in whole blood was due to CsA, M1, and M17, indicating that other RIA-NS reactive metabolites did not accumulate in blood. Serum creatinine concentrations were also measured before and after treatment in each of the animals. For rats treated for seven days the mean creatinine concentration pretreatment (0.60 ± 0.11 mg/dL) did not differ from the mean concentration posttreatment (0.60 ± 0.14 mg/dL). For animals treated 14 days, however, the mean pretreatment (0.40 ± 0 mg/dL) and posttreatment (0.77 ± 0.21) concentrations of serum creatinine were significantly different ($P < .05$).

DISCUSSION

CsA and metabolite concentrations in rat blood differ significantly from concentrations previously determined in blood from CsA-treated patients. In rat blood, we found that CsA concentrations predominate over the metabolites, while in blood from renal allograft recipients M17 has been found to predominate.[13] Similar to finding by Maurer et al,[11] we have also shown that M1 is the major metabolite in the rat. HPLC fractionation studies show, in addition, that other metabolites were not present in significant amounts in blood even when a high dose of CsA was administered to spontaneously hypertensive rats. Comparison of oral and SC treatments resulted in similar concentrations of primary metabolites, but lower concentrations of CsA with oral treatment. This effect of oral administration could be explained by a reduced bioavailability of CsA, as reported by Wassef et al,[17] coupled with an increase in liver metabolism of CsA when adsorbed through the gastrointestinal tract. Even with oral administration, however, CsA levels still predominated over metabolite levels in rat blood. Tissue studies have also shown a ratio of metabolites to CsA in kidney and liver that is similar to the ratio in blood. A previous study of human blood and renal tissue also showed that the relative amounts of CsA and primary metabolites are similar in blood and tissue, although in man the M17 is present in renal tissue in higher concentrations that CsA.[13] Therefore, the relative proportions of CsA, M1, and M17 levels in blood reflect the levels in tissue in both rats and humans.

Differences in metabolism between humans and rats may be important in understanding the mechanism by which CsA causes nephrotoxicity. Although the major purpose of this study was to assess CsA and primary metabolite levels in blood and tissue, it is of interest to note that very high concentrations of CsA were present in rat blood and tissue without significant impairment in renal function as determined by serum creatinine. In therapeutic monitoring of transplant recipients, trough blood concentrations of CsA greater than 250 ng/mL are potentially toxic.[18] In contrast CsA concentrations averaged 3,500 to 4,000 ng/mL in the Wistar-Furth rats treated with 15 mg/kg/d. Even in the spontaneously hypertensive rat, a strain that is

reported to be sensitive to CsA nephrotoxicity, 75 mg/kg/d of CsA did not alter serum creatinine concentrations until 14 days of treatment. In these animals the CsA concentration in blood averaged 13,000 to 14,000 ng/mL. It appears, therefore, that CsA-induced nephrotoxicity in rats is associated with much higher concentrations of CsA than in man.

A possible explanation for the resistance of rats to CsA-induced nephrotoxicity include a metabolite involvement in the nephrotoxic process or a difference in renal physiology between rats and humans. Although blood metabolite levels in rats are low compared to CsA levels, it is not known whether an increase in metabolite levels would result in nephrotoxicity. In an attempt to answer this question, Ryffel and coworkers[19] administered M17 orally (20 mg/kg/d) and SC (10 mg/kg/d) to spontaneously hypertensive rats for 28 and ten days, respectively. Although nephrotoxicity was not observed, questions concerning the oral bioavailability of M17 and the lack of M17 measurements in blood and tissue from oral and SC animal groups clouds the interpretation of these results. The question of metabolite nephrotoxicity, therefore, remains unresolved. Work by other investigators have suggested that creatinine measurements are not a sensitive index of nephrotoxicity in rats and that inulin clearance may be a more sensitive measure of nephrotoxicity.[20] Even in this study, however, the CsA dose (50 mg/kg/d) used to reduce inulin clearance far exceeded the toxic dose in humans, indicating again the resistance of rats to nephrotoxicity.

In summary, significant differences in CsA metabolism exist between rats and humans. Further studies are needed to understand the potential involvement of metabolism in the nephrotoxic process and these studies may lead to a better understanding of the mechanism by which CsA treatment causes nephrotoxicity.

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