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The Clinical Significance of Cyclosporine Metabolites

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Cyclosporine (CsA) is extensively metabolized, with over 14 metabolites having been characterized to date. The confirmation of structure and purity is a prerequisite for studies involving CsA metabolites. Analytical techniques such as fast atom bombardment/mass spectroscopy (FAB/MS), tandem mass spectrometry (MS), ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) can be used for such purposes. *In vitro* experiments indicate that metabolites are considerably less immunosuppressive and toxic than CsA. *In vivo* studies have been hampered by sufficient quantities of metabolites and a suitable animal model. Preliminary results in the rat suggest that CsA metabolites are less immunosuppressive and toxic than CsA, although these results must be confirmed using a more suitable animal model. Present data indicate that the routine monitoring of metabolites is not warranted in transplant patients, although additional information is required to confirm these findings.

KEY WORDS: cyclosporine; nuclear magnetic resonance; mass spectrometry; pharmacokinetics; immunosuppression; renal transplantation.

Introduction

Cyclosporine (CsA) is extensively metabolized by the hepatic P-450 III A (1). This isoenzyme has also been called P-450 NF and is responsible for the metabolism of many hydrophobic compounds including nifedipine, cortisol, erythromycin, and quinidine. A second cytochrome, P-450 $hPCN_3$, has also been found to be involved in CsA metabolism (2). Over 14 metabolites resulting from the biotransformation of CsA have been chemically characterized to date (3–5). The role of these metabolites in overall immunosuppression and toxicity remains uncertain. This information will determine whether one or more of the metabolites should be monitored along with parent CsA as a guide for dosage adjustments to minimize toxicity while maximizing immunosuppression. In this review, we will attempt to address many of the controversial issues surrounding CsA metabolites including nomenclature, isolation, chemical characterization, immunosuppressive activity, and toxicity, as well as information gleaned from clinical studies.

Nomenclature, structure, biotransformation

The present nomenclature system for CsA metabolites is based in part on their HPLC retention times. This has resulted in a lack of a systematic approach to naming newly isolated metabolites. It has recently been proposed that the nomenclature of metabolites be revised and standardized to include information on their chemical structure (6). A summary of the major metabolites currently identified under the present and proposed nomenclature systems are listed in Table 1. In the proposed system, the amino acid at which the modification occurs is listed numerically. For modifications consisting of hydroxylation, only the site of the modification is listed, *e.g.*, AM1. For demethylation, an N will follow the numerical value for that amino acid. The notations for other modifications are listed in Table 1. The A preceding each number indicates that the compound is a metabolite of CsA. This nomenclature system also provides guidelines for naming the metabolites of other cyclosporine analogues such as Cyclosporine G (Nva^2-Cyclosporine). Metabolites of this analogue will be preceded by a G. For the sake of clarity, only the new system of nomenclature will be used throughout the manuscript. Reference to the old nomenclature system can be made in the comparison listed in Table 1.

The proposed biotransformation of the metabolites is shown in Figure 1. The scheme is based primarily on chemical structure rather than direct proof. A recent study in which AM1 was administered to rabbits followed by monitoring of resulting metabolites provides the first direct evidence in support of the proposed scheme (7) (see *In Vivo* Studies).

Isolation and chemical characterization

CsA metabolites have been isolated from the bile and urine of a number of animal species as well as

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TABLE 1
Structure of Characterized CsA Metabolites

Metabolite Nomenclature							
New	Old	AA_1[a]	AA_4		AA_6	AA_9	Other
CsA	CsA	H	CH_3	H	H	H	
AM1	M-17	OH	CH_3	H	H	H	
AM1c[b]	M-18	OH	CH_3	H	H	H	AA_1: Cyclization
AM1DI[c]	Dihydro-M-17	OH	CH_3	H	H	H	AA_1: Saturated
AM4N	M-21	H	H	H	H	H	
AM9	M-1	H	CH_3	H	H	OH	
AM19	M-8	OH	CH_3	H	H	OH	
AM1DI9	—	OH	CH_3	H	H	OH	AA_1: Saturated
AM14N[d]	M-25	OH	H	H	H	H	
AM49	M-10	H	CH_3	OH	H	OH	
AM4N9	M-13	H	H	H	H	OH	
AM69	M-16	H	CH_3	H	OH	OH	
AM1c9	M-26	OH	CH_3	H	H	OH	AA_1: Cyclization
AM4N69	M-9	H	H	H	OH	OH	
AM1A[e]	M-203-218	COOH	CH_3	H	H	H	
AM1S[f]	—	SO_4	CH_3	H	H	H	AA_1: Sulfation at β-Carbon

[a]AA_1: Amino acid 1; [b]c: Cyclization; [c]DI: Dihydro; [d]N: Demethylation; [e]A: Carboxylic acid; [f]S: Sulfation.

man using various HPLC methods (5,8–19). A major problem with the early work on CsA metabolites was a lack of procedures for the confirmation of purity and structure of the isolated metabolites. Assessment of the former was based on the isolated metabolite eluting as a single symmetrical peak by HPLC. Identification was based, at best, on comparison of the retention time of the isolated metabolite to that of standard metabolite preparations obtained from the drug manufacturer. Over the past few years, a number of additional analytical procedures have been introduced which have aided in the characterization of CsA metabolites. A summary of these studies is listed in Table 2. These procedures include amino acid analysis, fast atom bombardment/mass spectroscopy (FAB/MS), tandem mass spectrometry, and proton and ^{13}C-nuclear magnetic resonance (NMR). Each one of these procedures has advantages and limitations. For example, FAB/MS can give reliable information on molecular weight and

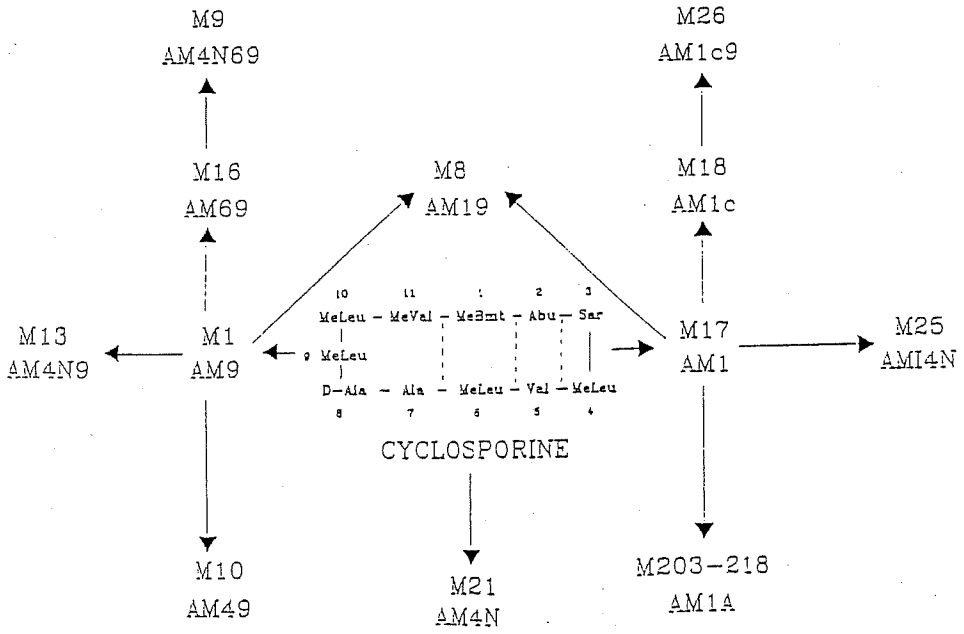

Figure 1—Biotransformation of CsA metabolites.

TABLE 2
Characterization of CsA Metabolites

Investigator	Reference	Isolation		Methodology
		Species	Medium	
Maurer (1984)	(8)	Rat, dog, man	Feces, bile, urine	FAB/MS, ^{1}H-NMR, ^{13}C-NMR, Amino acid hydrolysis
Hartman (1987)	(9)	Rabbit	Bile	HPLC, FAB/MS
Rosano (1986, 1987)	(10,11)	(Metabolites obtained from Sandoz)		HPLC
Wong (1988)	(13)	Man	Bile, blood, urine	HPLC, FAB/MS
Wallemacq (1989)	(14)	Man	Bile	HPLC, FAB/MS
Wang (1989)	(15)	Man	Bile	FAB/MS, ^{1}H-NMR, GC/MS, Amino acid analysis
Yatscoff (1990)	(16)	Man	Urine	HPLC, FAB/MS, ^{1}H-NMR, ^{13}C-NMR
Bowers (1990)	(22)	Man	Bile	HPLC, FAB/MS, Tandem MS

purity of the metabolite, but its utility in identification of the metabolite in question is limited since many of the metabolites have the same molecular weight. One-dimensional proton NMR can provide limited information on the site of the structural modification. This procedure can only reliably detect modification of the ηCH_3 of amino acid 1 and demethylation of amino acid 4. Two-dimensional proton NMR and ^{13}C-NMR using computer-assisted analysis can provide the most detailed information on the site of chemical modification of the metabolites. The major disadvantage of the latter two procedures is that a minimum of 1 mg of purified metabolite is required for analysis, which is 10-fold greater than that required for FAB/MS and one-dimensional ^{1}H-NMR. Examples of the spectra obtained by these procedures for CsA and its monohydroxylated metabolite, AM1, are shown in Figures 2 and 3.

Tandem mass spectrometry has played an increasingly important role in structural determinations of peptides (20,21). The technique can select a protonated molecular species produced with FAB or with electrospray/ionspray, fragment it by collision with a neutral gas molecule, and analyze the mass of the product ions using a second mass spectrometer. The pattern of product ions obtained is characteristic of the amino acids contained in the peptide and their sequence from the N- and C-terminal ends. Cyclosporine presents a difficult challenge since cyclic peptides frequently give rise to numerous ions because there are no defined terminal amino acids. Using analogues of CsA, identification of the amino acids giving rise to the various product ions has been accomplished (22). By studying the patterns of mass shifts of product ions in metabolite spectra, the modified amino acid can be determined. For example, the MS/MS patterns for CsA and A4N are shown in Figure 4. Note that the m/z 199 ion present in the CsA spectrum is absent in the AM4N spectrum, and that an m/z 185 ion has appeared. Ions from m/z 298 and 322 in the CsA spectrum have also shifted to 284 and 308 in the A4N spectrum. The common amino acid in these three ions is ^{4}MeLeu, and the loss of 14 daltons is consistent with loss of a methyl group. Using this approach, a structure can be obtained on a few picomoles of metabolite. A strength of the MS/MS technique is

TABLE 3
In Vitro Immunosuppressive Activity — Study Summary

Investigator	Reference	Assay[a]
Rosano (1986)	(10)	MLR, PHA, ConA, PWM
Rosano (1987)	(11)	MLR-IL2, PHA, ConA, CTL
Wonigeit (1987)	(24)	MLR, CD3
Ryffel (1988)	(25)	MLR, PHA, ConA, CD3
Zeevi (1988)	(26)	MLR, PLT, ConA
Wong (1988)	(13)	MLR, ConA
Wallemacq (1989)	(14)	MLR, ConA, PHA, CD3
Heidecke (1989)	(27)	Jurkat-IL2
Yatscoff (1989)	(16)	MLR
Yatscoff (1990)	(5)	MLR, PHA

[a]MLR: Mixed lymphocyte reaction; PHA, ConA, PWM: Mitogen stimulated assays; CTL: Cytotoxic T cell assay; CD3: Anti CD3 stimulation; PLT: Primed lymphocyte test; Jurkat-IL2: Interleukin 2 release by Jurkat cells.

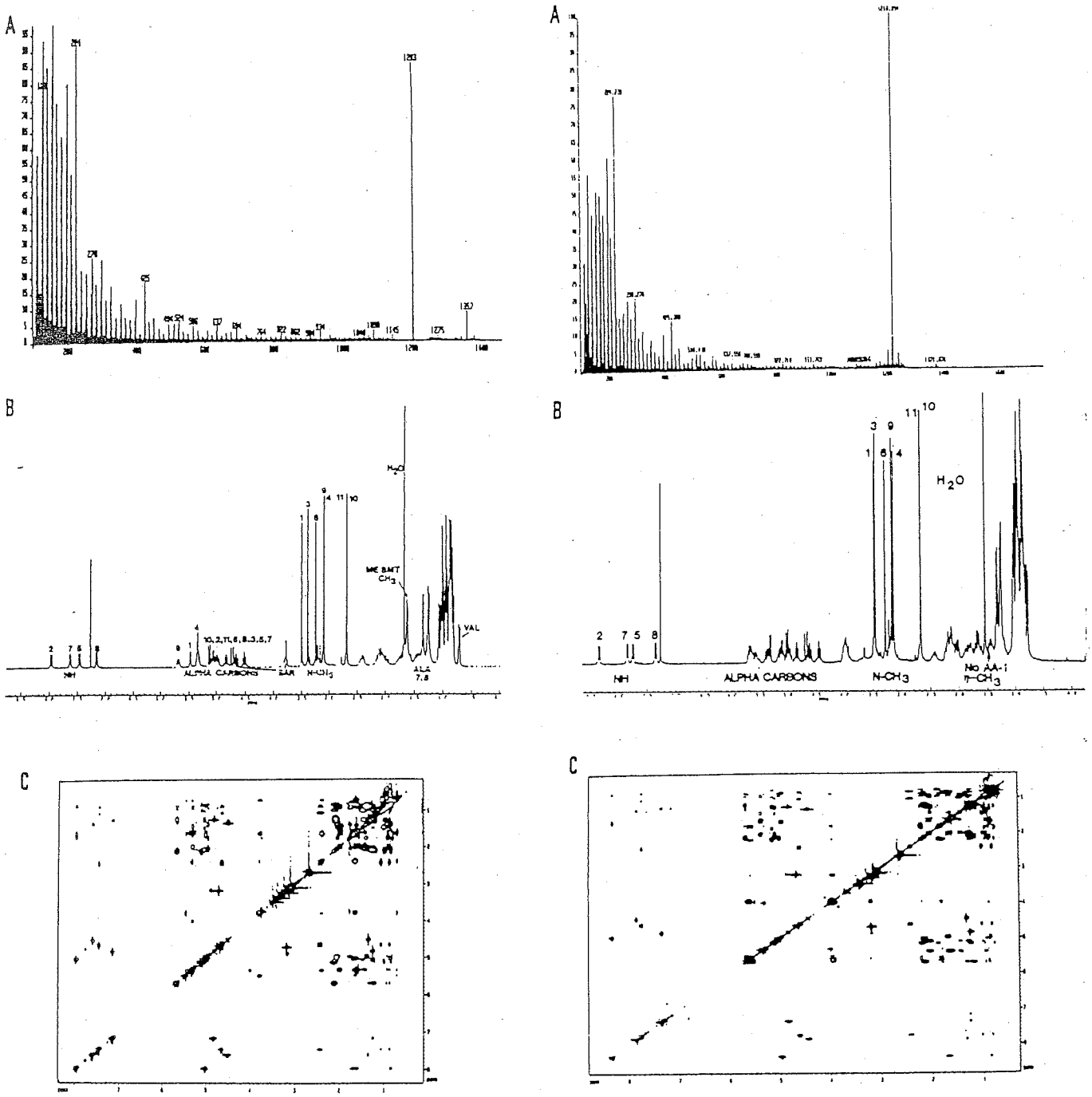

Figure 2—Comparison of FAB/MS (A), one-dimensional ^{1}H-NMR (B), two-dimensional ^{1}H-NMR (C) of CsA (left side) and its monohydroxylated metabolite, AM1 (right side).

that structural information can be obtained from mixtures of peptides because of the selection of a particular molecular mass for collision. A disadvantage is that the structure is not as definitive as that obtained from ^{13}C-NMR, and the technique is limited in its ability to assess purity above 95%.

In vitro immunosuppressive activity

The immunosuppressive activity of CsA metabolites has been studied by a number of investigators using a variety of *in vitro* test systems (5,13,14,16–18,23–27). These include primary and secondary mixed lymphocyte reaction (MLR), mitogen-stimulated systems as well as the effect of metabolites on the release of interleukin-2 from a Jurkat cell line. A listing of these studies can be found in Table 3. The purity of the metabolite used as well as the variable response of the culture techniques has been a source of controversy in these studies. In a recent study, many of these problems are overcome by testing the immunosuppressive activity of me-

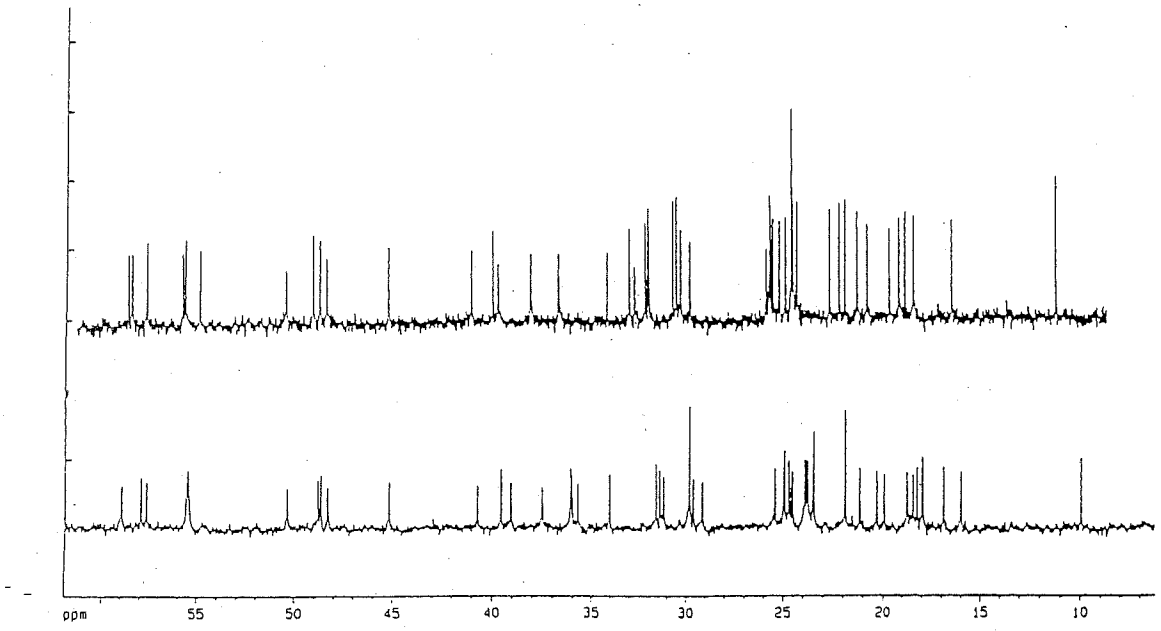

Figure 3—Comparison of the ^{13}C-NMR spectra of CsA (bottom) and its monohydroxylated metabolite, AM1 (top).

tabolites with known structure and purity in three separate *in vitro* assay systems (5). A summary of the immunosuppressive activities of the eight metabolites tested is shown in Table 4 (5).

From the above studies, the following conclusions can be made regarding the immunosuppressive activities of CsA metabolites:

(i) Several metabolites retain the immunosuppressive properties of CsA although to a much lesser extent.
(ii) The amount of activity is affected by the site of modification.
(iii) The primary CsA metabolites, AM1, AM9, and AM4N, are the most active metabolites identified so far. AM1 is the most active, having 10–20% activity of that of the parent drug.
(iv) Metabolite immunosuppressive activity decreases with increasing polarity.
(v) *In vivo* animal studies are needed to confirm these findings.

In vitro toxicity of metabolites

Studies on the *in vitro* toxicity of CsA metabolites have used both established and primary cell cultures. The porcine proximal renal epithelial cell

TABLE 4
Immunosuppressive Activities of CsA Metabolites in Three Separate *In Vitro* Assay Systems[a]

	Concentration of Metabolite (μg/L) Required for 50% Inhibition of ^{3}H-Thymidine Incorporation (IC_{50})[b]					
Metabolite	Primary MLR[c]		Secondary MLR[c]		PHA[c]	
AM19	>2,000	(<0.007)[d]	8,933 ± 1,508	(0.001)	>20,000	(<0.026)
AM1c9	>2,000	(<0.001)	7,270 ± 870	(0.002)	>20,000	(<0.026)
AM4N9	1,667 ± 189	(0.009)	7,250 ± 2478	(0.002)	>20,000	(<0.026)
AM1	170 ± 7	(0.089)	83 ± 17	(0.16)	20,700 ± 3,600	(0.025)
AM1DI	353 ± 81	(0.042)	527 ± 107	(0.02)	11,200 ± 4,200	(0.046)
AM9	157 ± 19	(0.095)	90 ± 24	(0.14)	5,630 ± 120	(0.092)
AM1c	1,500 ± 400	(0.01)	520 ± 106	(0.025)	11,900 ± 2,400	(0.044)
AM4N	183 ± 12	(0.082)	367 ± 26	(0.035)	7,300 ± 2,400	(0.071)
CsA	15 ± 3	(1.0)	13 ± 2	(1.0)	520 ± 100	(1.0)

[a]Taken from Ref. 5.
[b]Results are expressed as mean ± SD of three measurements done on separate days.
[c]Abbreviations defined in footnote to Table 3.
[d]Relative potency of metabolite to CsA (CsA = 1.00) is listed inside parentheses.

TABLE 5
In Vitro Toxicity of CsA Metabolites in a Porcine Renal Epithelial (LLC-PK$_1$) Cell Line[a]

Metabolite	Cell Growth[b]	DNA Synthesis	RNA Synthesis	Protein Synthesis
CsA	2,000 (1.00)	2,500 (1.00)	6,000 (1.00)	3,800 (1.00)
AM1	25,000 (0.08)[c]	>25,000 (<0.10)	>25,000 (<0.24)	>25,000 (<0.15)
AM4N	11,000 (0.18)	15,000 (0.17)	12,000 (0.50)	12,000 (0.32)
AM9, AM19, AM1c9, AM4N9, AM1c, AM1DI	>25,000 (<0.08)	>25,000 (<0.10)	>25,000 (<0.24)	>25,000 (<0.15)

[a]Taken from Ref. 31.
[b]Results expressed as the mean concentration of metabolite (μg/L) in duplicate experiments required for 50% inhibition of function (IC_{50}).
[c]Results in parentheses indicate potency relative to CsA (CsA = 1.00).

line (LLC-PK$_1$) has been extensively used to study CsA toxicity *in vitro* (28–30). Cole *et al.* (28) investigated the effect of two primary (hydroxylated) CsA metabolites, AM1 and AM9, on the metabolism and growth of this cell line as well as a primary rat mesangial cell line. These metabolites, up to a

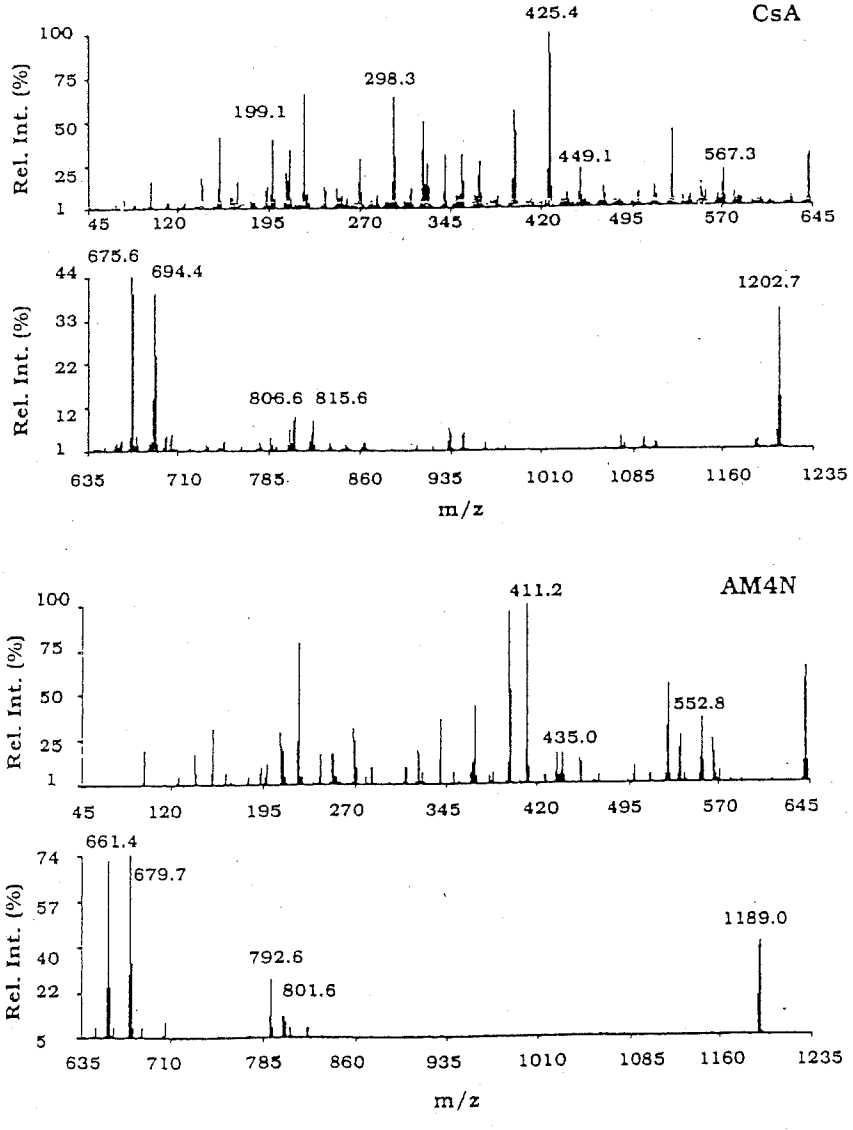

Figure 4—Comparison of the MS/MS pattern for CsA and AM4N.

TABLE 6
CsA Metabolite Concentration in Blood and Urine of Sprague Dawley Rats After 3 Days of Treatment (10 mg/kg/day) with Either CsA or AM1[a]

	Blood Concentration (μg/L)			Renal Excretion (μg/day)		
Treatment	CsA	AM1	AM9	CsA	AM1	AM9
CsA	1228 ± 685	<25	47 ± 17	5.48 ± 1.92	4.44 ± 0.79	6.13 ± 0.99
AM1	ND	36 ± 6	ND	ND	101.24 ± 18.02	ND

[a]Studies performed at the Albany Medical Center.
ND: None Detected.

concentration of 10,000 μg/L, exerted a minimal effect on DNA, RNA, or protein synthesis. In contrast, CsA at 500 μg/L resulted in a significant inhibition of these metabolic functions in both cell lines (28). In a more recent report, the *in vitro* toxicity of eight CsA metabolites was tested in the same cell line (LLC-PK_1) (31,32). None of the metabolites was as effective as CsA in inhibiting cell growth, DNA, RNA, or protein synthesis with the majority of them exhibiting activity of less than 10% of that of CsA (Table 5). The exception to this was the demethylated metabolite AM4N which exhibited a potency of 16–50% of CsA for the various metabolic parameters examined. It is important to note that the monohydroxylated metabolites, AM1 and AM9, which have been shown to have slightly greater immunosuppressive activity than AM4N *in vitro*, were found to be not toxic. This suggests that the *in vitro* immunosuppressive activity and toxicity of metabolites may be dissociated. Further *in vivo* studies are needed to confirm this finding.

Wilson and Hreniuk (33) have previously used a model system of primary cultures of defined renal epithelial cells derived from individually microdissected rabbit renal tubules to study the toxic effects of CsA. Toxicity was determined by cell survival, as indicated by nigrosine uptake. These studies indicated that proximal convoluted (PCT) and straight (PST) tubules were sensitive to CsA, while cells from the thick ascending limbs of Henle and convoluted collecting tubules (CCT) showed much less sensitivity to CsA. Both a dose-response and a time-related response were observed with CsA. Further studies with both rabbit and human fetal PCT cells have shown a marked decrease in protein synthesis which may be related to calcium-mediated protease activity. In this system, human cells in culture were shown to be more sensitive than rabbit cell cultures to the toxic effects of CsA. Studies in human cells were extended to metabolites, and several were found to cause cell death including AM1c, AM1A, AM4N, and AM9 (39). Although cell death has been questioned as an end point, it does provide data about toxic effects. Studies are underway to further characterize the mechanism of toxicity.

Studies investigating the effect of CsA metabolites on hepatocytes have also been reported (34–38). Boelsterli *et al.* (34) have shown in primary rat hepatocyte culture that CsA can decrease bile acid conjugation, and at very high concentrations (>1,000 μg/L), can decrease protein synthesis. No effects

TABLE 7
Pharmacokinetic Parameters of CsA and its Metabolite AM1 in Rabbits[a]

	Rabbit Number	Clearance (Cl) (mL/min/kg)	Volume of Distribution (Vdss) (L/kg)	Half-Life Terminal ($T_{\frac{1}{2}}$ Terminal) (h)
CsA[b]	1	12.47	2.800	3.99
	2	8.85	1.206	2.54
	3	12.23	1.582	2.77
Mean ± SD		11.18 ± 2.02	1.863 ± 0.833	3.10 ± 0.28
AM1[b]	4	14.92	1.840	1.94
	5	9.68	1.071	2.11
	6	8.62	1.396	2.61
Mean ± SD		11.07 ± 3.37	1.436 ± 0.386	2.22 ± 0.35

[a]Taken from Ref. 7.
[b]CsA and AM1 were administered at a dose of 1 mg/kg IV over a period of 3 min.

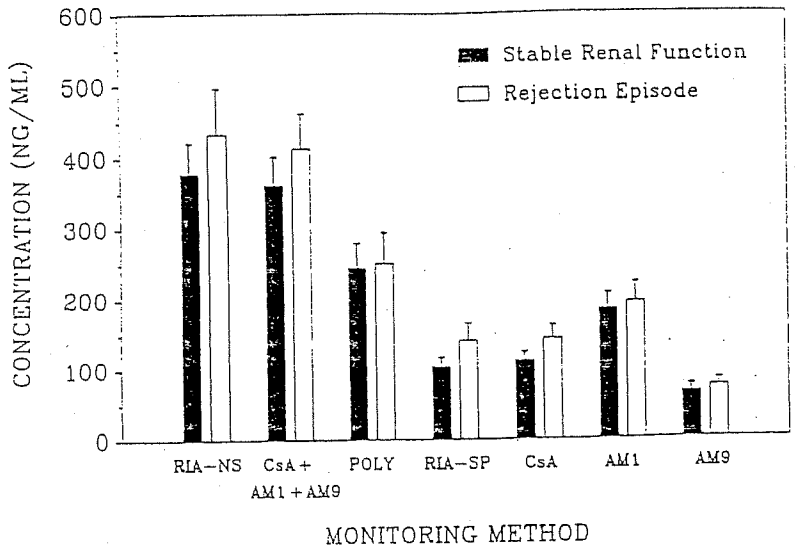

Figure 5—Comparison of CsA and metabolite monitoring for renal transplant patients with and without rejection episodes. Specific and nonselective monitoring of trough blood from 10 patients with graft rejection episodes occurring between 7 and 83 days post-transplant were compared with monitoring during the same time period in 8 patients with stable renal function. Whole blood samples collected 7 days prior to the rejection episode were analyzed by the Sandoz monoclonal specific (RIA-SP), nonspecific (RIA-NS), and polyclonal (POLY) RIA as well as an HPLC method for CsA, AM1, and AM9 (10). Data are presented as the mean plus standard error of the mean. Statistical analysis showed no significant difference ($p > 0.05$) between the two groups with any of the methods.

TABLE 8
A Summary of HPLC Methods Used for Clinical Measurements of CsA Metabolites

Investigator	Reference	Sample Treatment	HPLC/Column Conditions	Metabolites Measured	Transplant Indications
Rosano (1986) Rosano (1986) Rosano (1988)	(11) (10) (54)	Organic extraction	CN/Isocratic	AM9, AM1, AM4N	Kidney
Lensmeyer (1988) Lensmeyer (1987)	(42) (55)	Solid phase	CN/Isocratic	AM9, AM19, AM1, AM1c, AM4N, AM14N, AM1c9, AM1A	Heart, liver, bone marrow
Yee (1988)	(56)	Protein precipitation	C8+C18/Isocratic	AM1	Bone marrow
Wang (1988)	(57)	Organic extraction	C18/Gradient	AM9, AM1, AM1c, AM4N	Heart, liver, kidney, bone marrow
Awni (1988) Awni (1989)	(58) (43)	Solid phase	CN/Isocratic	AM9, AM1, AM4N	Kidney
Roberts (1988)	(59)	Organic and solid phase	C18/Isocratic	AM1	Kidney, pancreas
Christians (1988)	(60)	Solid phase	C8 (2 columns in tandem)/Gradient	AM1, AM1c, AM4N, AM9, AM19, AM4N69, AM14N, AM4N9, AM69	Liver
Bowers (1987)	(61)	Organic extraction	C18/Gradient	AM1, AM9, AM1c, AM4N	Kidney, liver

were observed for AM1. Studying a wider range of metabolites in primary rat hepatocyte culture, inhibition of conjugation of cholate with taurine was observed for AM1, AM9, and AM1c (35). No effect was observed on LDH leakage, amino acid uptake, ATP concentrations, or protein synthesis. Earlier studies with CsA had shown an increase in LDH leakage and an increase in 2-deoxyglucose uptake (36). Riegel *et al.* (37,38) have demonstrated altered glycogen metabolism both in liver tissue and primary hepatocytes isolated from rats treated with CsA. No specific studies on metabolites were performed, but this may represent another area of study to determine metabolite toxicity.

In vivo studies

Preliminary studies investigating the nephrotoxic properties of CsA metabolites *in vivo* have been reported (25). AM1, when administered I.P. to rats at a dose of 10 mg/kg/d for 28 days, resulted in no morphological or biochemical evidence of nephrotoxicity (25). In contrast, a similar dose of CsA administered I.P. resulted in a significant decrease in creatinine clearance and renal morphological changes consistent with CsA nephrotoxicity. Although the doses of CsA and AM1 were similar, the average concentration of the metabolite in whole blood was less than half of CsA. It has recently been reported that pure AM1, AM1c, AM4N, and AM1A do not produce immunosuppression or nephrotoxicity when administered subcutaneously to SH rats for 10 days at 50 mg/kg/d (40). However, the results of this study were clouded by the fact that the area under the curve (AUC) for the metabolites, in particular AM1, was significantly less than CsA. The lack of activity of metabolites may be due in part to low blood levels.

A significant difference in CsA metabolism between humans and the rat model has previously been reported, and must be considered in the interpretation of *in vivo* experiments with the latter model. After 14-day treatment with CsA (15 mg/kg/d s.c.), the CsA concentration in rat blood and tissue exceeded that of metabolites by 5 to 7 fold with AM9 as the major metabolite (41). In contrast, AM1 is the major blood and tissue metabolite in renal transplant patients and in humans the AUC for metabolites exceeds that of CsA (10,42,43). These species differences may be due to a difference in metabolite production, elimination, or both. Initial results of a study comparing CsA and primary metabolite concentrations in blood and urine from Sprague Dawley rats after 3-day treatment (10 mg/kg/d) with CsA and AM1 have shown a rapid renal clearance of AM1 associated with low blood concentrations of the metabolite. Table 6 compares blood concentration and urine excretion of CsA, AM1, and AM9. The inhibition of IL-2 production by lymphocytes harvested from spleen and lymph nodes is now under investigation. Metabolite disposition of CsA and metabolites in the rat may not, however, allow a valid extrapolation of results to humans and an alternate animal model may be needed to evaluate biological activity *in vivo*.

The rabbit may be such a model. It has previously been shown that AM1 is the major metabolite found in the blood and tissues of rabbits administered CsA, just as it is in renal transplant patients (44). When AM1 was administered to rabbits at a dose of 1.0 mg/kg, the mean pharmacokinetic parameters were not significantly different from those obtained for CsA (Table 7) (7). In bile and urine obtained from rabbits administered AM1, metabolites AM19, AM1c,and AM1c9 were found, indicating that AM1 is further metabolized (7). These data support the proposed biotransformation pathways for AM1 (Figure 1). Rabbits, when administered CsA I.V. at doses as low as 2.5 mg/kg/d for 30 days, have also been shown to exhibit the changes associated with chronic CsA nephrotoxicity (interstitial fibrosis and arteriolopathy) found in humans (45). Ten-fold higher dosages of CsA are required to induce such changes in the rat (46). Further *in vivo* studies are planned with the rabbit to investigate both the immunosuppressive and toxic activities of metabolites.

Clinical studies

Clinical studies using immunoassay methods have yielded only limited information concerning the concentration and potential clinical significance of CsA metabolites (47–50). The nonselective immunoassay methods utilize polyclonal or monoclonal antibodies that preclude the selective measurement of metabolites due to varying immunocross-reactivity with the diversity of metabolites present in blood (51,52). Selective measurement of metabolite concentration in clinical samples has been performed in several transplant centres in order to determine blood levels, tissue distribution, and potential correlation of metabolites with clinical events. Table 8 summarizes methods used in the specific measurement of metabolites in clinical samples. HPLC methods used in these studies vary in sample treatment technique, chromatographic conditions, and source of metabolite calibrators. In general, these methods are more technically demanding than immunoassay, and their proficiency and selectivity have not yet been thoroughly evaluated between laboratories. With refinements in analytical technique, additional metabolites have been found to coelute chromatographically in some of these methods (53). Currently, however, these HPLC methods provide the most specific measurement of metabolites.

High-circulating concentrations of primary metabolites in trough blood were initially reported for renal transplant patients (55) and have been confirmed by multiple centres for other transplant indications (10,42,54–61). In contrast to rat studies that showed only a minor amount of metabolites in blood (41), the total concentration of metabolites in blood

from CsA-treated patients exceeds that of the parent drug. In addition to the major metabolite, AM1, other metabolites including AM9, AM19, AM1c, AM4N, AM14N, AM1c9, AM1A as well as structurally unidentified metabolites have been detected in blood from CsA-treated patients. Pharmacokinetic studies have further shown both a significant presence of metabolites during the entire dosing interval and a difference in pharmacokinetic parameters between CsA and the metabolites (43,58). The correlation between the $T_{\frac{1}{2}}$ of CsA and that of AM1 and AM9 suggests that these metabolites are rate limiting for parent drug elimination, and the more rapid renal clearance of AM1, AM9, and AM4N versus CsA indicates a greater effect of renal function on metabolite elimination. Serial trough profiles of metabolites (54) as well as periodic pharmacokinetic studies (43) during the first four months post renal transplant both showed no relative changes in the concentration of CsA and its primary metabolite. Clinical studies, therefore, indicate a relatively high concentration of metabolites in blood and suggest a significant exposure of peripheral tissue to these biotransformation products.

The actual distribution of metabolites in potential target tissue may be important in assessing biological activity. Cellular distribution was first evaluated in blood where metabolites partition between plasma and blood cell. Initial HPLC studies revealed a greater cellular binding of AM1 and AM9 as compared with CsA and AM4N (10). A more recent study (62) with a number of additional metabolites has shown a complex interplay of metabolite partitioning that is dependent on hematocrit, temperature, metabolite concentration, and structure. Overall, however, AM9, AM19, AM4N69, AM49, AM69, and AM1 preferentially partition into the cellular fraction while AM4N9, AM1c, AM4N, AM14N, AM1c9, and AM1A remain primarily in the plasma. Blood distribution studies indicate significant cellular binding of metabolites, and at a practical level, the variability in partitioning dictates the use of whole blood for clinical monitoring of CsA and its metabolites. In studies with human kidney tissue obtained from CsA-treated patients, a high concentration of AM1 was determined along with lesser concentrations of CsA, AM9, and AM4N (10). The relative CsA and metabolite concentration in renal tissue paralleled the pattern in blood. Postmortem examination of adipose, kidney, liver, muscle, pancreas, lung, spleen, and brain tissue has revealed up to a 53-fold higher concentration of CsA and metabolites in tissue compared to blood (42). Again, AM1 predominated in all tissues with the exception of adipose and pancreas where CsA was in highest concentration. The biological importance of metabolite distribution in tissue has not yet been established. Metabolite binding to cyclophilin has, however, been reported and an association between cyclophilin binding and immunosuppressive activity has been proposed (63).

A significant correlation of metabolite concentration with clinical events would provide the strongest practical justification for monitoring of specific metabolites in routine practice. Clinical studies to test the correlation of specific metabolites with clinical events such as graft rejection and nephrotoxicity are limited in both number and conclusion. *In vitro* culture studies suggest that the primary metabolites, especially AM1, have the ability to suppress IL-2 production and, therefore, contribute up to 20% of the immune suppression seen in the CsA-treated patient. Is it necessary, however, to monitor metabolites to ensure adequate immunosuppression in the patient? In a comparison of CsA and primary metabolite levels in blood from renal allograft recipients with and without rejection episodes, the metabolite measurements did not provide any additional predictive value in the clinical management of transplant rejection episodes (Figure 5). The absolute and relative concentrations of CsA and metabolites did not differ significantly in the two groups of patients. Specific monitoring of the major immunosuppressive agent, CsA, is currently recommended (4) and additional monitoring of the metabolites with immunosuppressive activity may not be needed to routinely evaluate dosage or patient compliance. Elevated metabolite concentration in blood, however, has been observed during nephrotoxic episodes in some renal (54) and liver (64) transplant patients, and questions have been raised concerning the clinical significance of these findings. To address these questions, the toxic properties of metabolites are being studied (see *In Vitro* Toxicity of Metabolites) and further clinical investigations are needed to determine the predictive value of specific metabolite measurements in nephrotoxicity.

In summary, the clinical studies with specific HPLC methods have yielded preliminary information on metabolite pharmacokinetics and tissue distribution. Further clinical studies and animal investigations with purified metabolites are needed prior to any recommendation for routine clinical monitoring of specific metabolites.

Conclusions

Although further investigation is required, the following conclusions can be made on CsA metabolites based on the available data.

(i) The confirmation of structure and purity is a prerequisite for studies involving CsA metabolites. Analytical techniques such as FAB/MS, tandem MS, ^{1}H- and ^{13}C-NMR can be used for such purposes.

(ii) *In vitro* experiments indicate that metabolites are considerably less immunosuppres-

sive and toxic than CsA.

(iii) *In vitro* experiments suggest that the immunosuppressive and toxic properties of metabolites may be dissociated.

(iv) *In vivo* studies with CsA have been hampered by the lack of sufficient quantities of metabolites and a suitable animal model.

(v) Preliminary results in the rat suggest that metabolites are less immunosuppressive and toxic than CsA. These studies must be confirmed using a more suitable animal model such as the rabbit.

(vi) The results of studies to date provide no support for the routine monitoring of metabolites.

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