BIOLOGICAL ACTIVITY OF CYCLOSPORIN A AND CYCLOSPORIN G METABOLITES

A Thesis Submitted to the
Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By

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Cyclosporine (CsA) is extensively metabolized to yield a number of metabolites. It is presently not known whether only parent drug or parent drug plus one or more of its metabolites should be monitored in patients receiving the drug to allow appropriate dosage adjustments to be made to maximize immunosuppression while minimizing toxicity. To answer this question, the immunosuppressive and toxic effects of CsA metabolites were investigated. CsA metabolites were isolated from urine obtained from renal transplant recipients and bile from liver transplant recipients using HPLC. In addition, metabolites of CsG, a potentially less toxic analogue of CsA, were also isolated. In most cases, the structure and purity of the isolated metabolites were determined by fast atom bombardment mass spectrometry and proton- and ^{13}C-nuclear magnetic resonance. The immunosuppressive activities of the isolated CsA and CsG metabolites were investigated using three different in vitro assay systems. None of the CsA metabolites were as immunosuppressive as the parent drug. The primary metabolites of CsA (M-17, M-1, and M-21) were 10 to 15% as immunosuppressive as CsA, whereas the other metabolites examined were substantially less. Additionally, pharmacokinetic studies revealed no differences in the pharmacokinetics of CsA and its major metabolite, M-17. CsG metabolites were found to be generally less than 10% as active as the parent drug. The in vitro toxicity of CsA metabolites was examined in both a porcine epithelial cell line ($LLC\text{-}PK_1$) and a primary rabbit mesangial cell line. None of the metabolites examined were as

cytotoxic as CsA in the in vitro assay systems used. The majority of metabolites exhibited toxicity less than 10% of that of CsA, except for M-21, a demethylated metabolite, which exhibited a potency of 17 to 50% of CsA, depending on the parameter examined. Further, CsA metabolites were as equipotent to the parent drug in causing a concentration-dependent reduction in prostacyclin release (a potent vasodilatory substance) from mesangial cells. CsA had no effect on inhibiting endothelin release from these cells; however, M-26 and M-8 resulted in a significant increase in its production. In summary, this thesis has demonstrated that CsA metabolites are generally less biologically active than parent CsA. None of the metabolites examined were as immunosuppressive or toxic as the parent drug. Although further investigation is required, to date, the routine monitoring of CsA metabolites in addition to parent CsA is not required to allow appropriate dosage adjustments to be made in the majority of transplant recipients.

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ABBREVIATIONS

AUC	Area under the concentration curve
Cl	Clearance
C_{max}	Maximum concentration
ConA	Concanavalin A
cpm	Counts per minute
CsA	Cyclosporin A (Cyclosporine)
CsC	Cyclosporin C
CsG	Cyclosporin G (Norvaline-Cyclosporine)
CsH	Cyclosporin H
°C	Degrees Celsius
cm	Centimeter
CV	Coefficient of variation
Ci	Curie
d	Day
Da	Dalton
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
DMEM	Dulbecco's minimal essential medium
EDTA	Ethylenediaminetetraacetate
FAB/MS	Fast-atom bombardment mass spectrometry
fmol	Femtomole
g	Gram
>	Greater than
xg	Relative centrifugal force
GFR	Glomerular filtration rate
HBSS	Hank's buffered saline solution
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	Human leucocyte antigen
HPLC	High performance (pressure) liquid chromatography
hr	Hour
IC_{50}	Concentration resulting in 50% of function
IL-2	Interleukin-2

i.p.	Intraperitoneal
i.v.	Intravenous
kg	Kilogram
<	Less than
L	Liter
MeBmt	Dimethyl-butenyl threonine
M-199	Medium 199
m/z	Mass over charge ratio
MH^+	Protonated molecular ion
µCi	Microcurie
µg	Microgram
µL	Microliter
µm	Micrometer (micron)
µmol	Micromole
µM	Micromolar
mg	Milligram
mL	Milliliter
mm	Millimeter
mmol	Millimole
mM	Millimolar
min	Minute
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
M	Molar
nm	Nanometer
N	Normal
NMR	Nuclear magnetic resonance
ppm	Parts per million
PGI_2	Prostaglandin I_2 (prostacyclin)
6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
pH	Negative log of hydrogen ion concentration
%	Percent
PHA	Phytohemagglutinin
pg	Picogram
p.o.	Oral administration

RIA	Radioimmunoassay
RNA	Ribonucleic acid
s.c.	Subcutaneous
SD	Standard deviation
$T_{\frac{1}{2}}$	Half-life
TxA_2	Thromboxane A_2
TCA	Trichloroacetic acid
U	International units
v	Volume
Vd	Volume of distribution
w	Weight

I. INTRODUCTION

Cyclosporine (CsA) is a member of a group of unique cyclic peptides of fungal origin, many of which possess potent immunosuppressive properties (1-3). The clinical use of CsA has had a major impact on the field of organ transplantation, drastically improving graft survival and decreasing morbidity over previously used immunosuppressant regimes which have included the use of compounds such as azathioprine or prednisolone (4,5). The potent immunosuppressive properties of cyclosporine were accidently discovered in the early 1970s by Borel et al (6). In 1970, a strain of fungi imperfecti was isolated from a soil sample obtained from Hardanger Vidda in northern Norway which was subsequently found to possess anti-microbial activity (6). This fungus was later identified as Tolypocladium inflatum Gams. In 1973 a metabolite of the fungus was purified and identified as cyclosporine and subsequently shown to demonstrate potent immunosuppressive properties. The first animal studies involving CsA were undertaken in 1974 to elucidate its immunosuppressive activity both in vitro and in vivo. These preliminary studies ultimately led to the use of CsA as a primary immunosuppressant in subsequent studies designed to examine its efficacy in both animal and human models of allograft transplantation. In 1976, Borel et al (7) reported the effective use of CsA in the prevention of allograft rejection in animals. In 1978, the first clinical trials were undertaken using the drug to prevent allograft rejection in cadaver kidney transplant recipients and in the

prevention of graft vs host disease in bone marrow transplantation (6,8,9).

Since the time of the first human studies, the clinical use of CsA has expanded tremendously. Today, it is used in a variety of different clinical situations. Besides being an integral component of immunosuppressive therapy in organ transplantation, its use has been expanded to encompass a wide range of clinical disorders, many of which have an autoimmune etiology (10-13).

A. CLINICAL USE OF CYCLOSPORINE

CsA has been used effectively to prevent allograft rejection in kidney, heart, and liver transplants (4,5,13,14,15,16), as well as in the prevention of graft vs host disease in bone marrow transplantation (17). Cyclosporine has not only significantly improved the initial and most probably long-term graft survival of allograft transplantation, but it has also mitigated the impact of various immunologic risk factors such as HLA mismatching and pre-transplant blood transfusions (13). In addition, it has reduced morbidity and patient hospitalization time. CsA is clearly established as the principal immunosuppressant in renal transplantation, where multicentre studies have demonstrated its superiority over previous agents in terms of both efficacy and safety. Graft survival in primary recipients is in the range of 85 to 95% following cadaveric or living donor transplantation, with a subsequent attrition rate of 2 to 3% per year (10). Outcome has improved, particularly in high risk groups such as the elderly, diabetic patients and sensitized or multiply grafted individuals. Transplantation has consequently become the treatment of choice for end-stage renal disease, providing significant medical and economic advantages over dialysis (10). The drug has also been used successfully in clinical trials for the treatment of several autoimmune diseases (10,13) including multiple sclerosis (10), uveitis (11), diabetes mellitus (12), psoriasis (18), and rheumatoid arthritis (19). In contrast, CsA has had little effect in treating diseases such as systemic lupus erythematosus and myasthenia gravis (13).

Despite the revolutionary impact on the field of organ transplantation, the use of cyclosporine is not without several side effects, many of which seriously hamper its clinical use (2,13,20-34). Nephrotoxicity is the side effect of most concern, affecting virtually all patients who receive the drug (20). Many of the complications are dose-dependent and can be reversed by reducing the dosage. For this reason it is recommended that CsA levels be monitored to minimize the toxic effects while still maintaining adequate immunosuppression (13,20). A detailed discussion of CsA nephrotoxicity will follow in a subsequent section.

Neurological side effects are common in patients receiving CsA and affect over 20% of those who receive the drug (2,13). Tremors and seizures are the primary dysfunctions associated with CsA neurotoxicity (2,21-23). Several other complications frequently occur, such as hirsutism resulting in excess hair growth on the face, arms, shoulders, and back (24); and hyperuricemia resulting in an increased incidence of gout (10). Gastrointestinal complications are also common and include stomach upset, nausea, and bloating (13). However, there is no direct toxic effect on the structure and function of the gastrointestinal mucosa. Furthermore, infectious complications are observed more frequently with CsA than with other immunosuppressive regimes such as steroids (25), and patients receiving CsA have an increased risk of developing malignant tumours (2). Hepatotoxicity is also a complication of CsA therapy, affecting almost half the patients who receive the drug (2,26,27). Biochemical alterations include increased serum bilirubin, alkaline

phosphatase, γ-glutamyl transferase, aminotransferase, and bile acids (13,26). In contrast, there is decreased bile flow and a slight decrease in serum albumin. Morphologically, choleostasis, vacuolization, and cell necrosis of the hepatic parenchyma are seen (26).

CsA can also affect the blood vessels, directly resulting in several vascular complications. The most serious consequence is hypertension (28). Other vascular complications include thrombosis, increased platelet aggregation, and alterations in prostaglandin production, all ultimately resulting in hypertension (28).

B. CsA NEPHROTOXICITY

In humans at least three patterns of CsA nephrotoxicity have been observed: 1) functional changes, 2) structural changes, both of which are dose-dependent and reversible by a reduction in the dose, and 3) irreversible chronic nephrotoxicity (20,25,29-37).

CsA-induced renal functional changes result in both tubular and vascular alterations (31-33). Tubule functional derangements include decreased serum magnesium and a mild increase in potassium and uric acid levels. These changes are due to alterations in reabsorption and excretion of these compounds (32,35). These tubule changes are often seen with therapeutic doses of the drug; however, they are fully reversible upon dose reduction and are of little clinical consequence. The functional changes occurring in the renal vasculature result from increased vasoconstriction of the afferent arterioles, decreased kidney perfusion, and decreased filtration, resulting in increased

serum creatinine and serum urea (32,36). Once again, these changes are reversible by dose reduction (31,32).

Morphological changes observed with CsA primarily affect the tubules and renal vasculature (32,33,37,38). Structural changes to the tubules are confined to the proximal tubule and consist of vacuoles, giant mitochondria, single cell necrosis, and microcalcification (32-34,37,38). These changes are reversible upon reduction of CsA dose. The vasculature changes are confined to the afferent arteriole near the glomerulus. Damage to the endothelial and smooth muscle cells in this location can lead to occlusion and obliteration of the arteriole, with collapse and sclerosis of the associated glomerulus. Other changes in addition to vascular occlusion and tubule atrophy include the presence of striped interstitial fibrosis and localized ischemia. These changes are irreversible and result in permanent damage to the kidney (33,34).

The mechanism by which CsA induces renal damage remains poorly understood due to a number of reasons. First, the techniques used for evaluating renal function in CsA-treated patients are relatively unsophisticated. The detection and monitoring of renal injury have usually been limited to determinations of serum creatinine (2,20,29-33). Secondly, the majority of clinical experience with CsA has been in renal transplant recipients (2,20,29,32). Such patients are unsuited to the clinical investigation of CsA nephrotoxicity because the co-existence of acute or chronic rejection makes CsA-induced derangements in renal structure and function difficult to isolate (32). Thirdly, attempts to develop an animal model of chronic

CsA nephrotoxicity, similar to that observed in humans, have had limited success (32).

It is generally thought that increased vasoconstriction of the renal vasculature is responsible for the functional changes observed in CsA nephrotoxicity. Results from early studies had suggested CsA itself was a vasoconstrictor, but subsequent studies have revoked this hypothesis (32,39). The alteration of renal prostanoid production is another theory which could explain the role of vasoconstrictory mechanisms in the development of CsA nephrotoxicity (32,33). The prostanoids are a group of potent vasoactive substances. Of particular interest are prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) which are vasodilators, and prostaglandin F_2 (PGF_2) and thromboxane A_2 (TxA_2) which are vasoconstrictory mediators (32). TxA_2 in particular is a potent vasoconstrictor; therefore very small increases in its concentration coupled with small decreases in PGE_2 or prostacyclin could lead to considerably increased vasoconstriction of the renal vasculature (32).

In animals the excretion of TxB_2 (the stable metabolite of TxA_2) is increased by CsA treatment (40-42); however, the excretion of 6-keto $PGF_{1\alpha}$ (the stable metabolite of prostacyclin) and PGE_2 is also increased (43,44). In contrast, in humans, TxB_2 excretion is often unchanged or depressed and the excretion of $PGF_{1\alpha}$ and PGE_2 may also be reduced (45,46). Since prostacyclin is produced by the vasculature endothelium, TxA_2 by the platelets and glomerular mesangium, and PGE_2 in the renal medulla and cortex, urinary excretion may not accurately reflect local rates of production (32). The excretion of prostanoids

is also affected by tubular reabsorption and secretion as well as by urine flow rate and acidity (32). Therefore prostanoid release from those tissues that produce them may provide more reliable information.

In renal tissue obtained from CsA-treated animals, TxB_2 release from isolated glomeruli or cortical slices was unchanged or increased (40,47). In other studies, the release of 6-keto-$PGF_{1\alpha}$ and PGE_2 from isolated glomeruli was reduced (48,49). In contrast, in animal endothelial cells grown in culture, basal release of 6-keto-$PGF_{1\alpha}$ was increased when high doses of CsA reduced cell cycle replication and viability. Human cells exposed to lower doses and shorter incubation times of CsA resulted in a depressed release of both basal and stimulated 6-keto-$PGF_{1\alpha}$ (48). In a recent study, CsA treatment was shown to alter prostanoid and thromboxane production by isolated kidney mitochondria (50). The results from the aforementioned studies suggest that there is a trend to a reduction in prostacyclin and PGE_2 production, while TxA_2 production may be increased in renal vascular tissue obtained from humans or animals exposed to CsA, although more experimentation is required to confirm this.

Another potential mechanism causing vasoconstriction of the kidney vasculature is the activation of renin within the vessel walls. In both dogs and rats, plasma renin activity was seen to be increased after treatment with CsA (32). In contrast, in humans, plasma renin activity was unchanged or even depressed by CsA therapy (32,38). However, inactive renin seems to be increased by treatment with CsA in both rats and in humans even though plasma renin activity was not elevated.

Recently it has been demonstrated that in addition to prostanoids, various cells produce other vasoactive substances including a novel peptide, endothelin (51,52). Endothelin is an acidic 21 amino acid peptide (2492 Da) which contains two sets of intrachain disulfide bonds. Its structure is similar to that of a group of peptide toxins found in snake venom (53). Endothelin is the most potent vasoconstrictor known to date, causing a strong and sustained vasoconstrictive response in most arteries and veins (52,54). Exogenous infusion of endothelin has been shown to cause systemic hypertension and long-lasting constriction of vessels both in vivo and in vitro. In CsA-treated rats, it was shown that endothelin levels were significantly increased over control animals (55). Furthermore, the effects of endothelin (decreased GFR and renal plasma flow) could be prevented by infusion of a rabbit anti-porcine endothelin antibody. These preliminary results suggest that endothelin may have a pivotal role in the pathophysiology of CsA-induced acute renal vasoconstriction and glomerular dysfunction.

As stated earlier, the animal models available to study CsA toxicity are limited (13,56-62). This has hampered attempts to focus on the mechanism(s) involved in CsA nephrotoxicity. The majority of studies to date have been carried out in rats. Most rodent models have failed to exhibit the chronic CsA nephrotoxicity seen in man even when the doses used were as high as 50 mg/kg/d orally for 90 days or at 25 mg/kg/d i.p. for 15 days (57-59). In contrast, a recent report in which Sprague-Dawley rats were administered CsA (25 mg/kg/d i.p. for 28 days) resulted in renal lesions similar to those observed in

chronic CsA nephrotoxicity in humans (60). It should be noted that all functional alterations observed in rat models only occur at pharmacological doses of the drug, levels which are much higher than those used clinically in humans (57-60). Moreover the vascular lesions seen in man, including arteriolopathy, have not been reproduced in any rodent model. The rabbit has been used less frequently as a model of CsA nephrotoxicity (58,61). A recent report from our laboratory describes chronic nephrotoxicity at therapeutic levels of the drug using a rabbit model. The alterations observed consist of both chronic functional and morphological changes consistent with the alterations observed in man (62). This model may prove useful in investigating the mechanisms of CsA nephrotoxicity.

The use of cell culture systems to study CsA nephrotoxicity has aided in gaining an understanding into the progression and course of its pathogenesis (63). Various cell lines and cultures, including primary cell cultures as well as established renal cell lines, have been used to study CsA toxicity (42,51,63-76). CsA nephrotoxicity has been widely studied using the LLC-PK_1 cell line (51,63-69). These cells were derived from porcine proximal tubule epithelial cells. They still retain most morphological and biochemical characteristics of epithelial tubule cells; however, they exhibit an altered hormone pattern (63). The advantage of using an established cell line is the ease with which large amounts of cells can be cultured for subsequent studies. Studies by Becker et al (64) initially reported that CsA inhibited the growth of LLC-PK_1 cells. Subsequent studies by Walker et al (65,66) demonstrated that CsA had significant effects on the

cellular metabolism of these cells. CsA at 10,000 μg/L significantly inhibited DNA and protein synthesis. Subsequent studies by Chan et al (69) have demonstrated that CsA inhibits glucose transport in this cell line (69).

Primary cell cultures have been used with much success to investigate CsA nephrotoxicity. Cultures have been obtained from rat epithelial cells (70), mesangial cells (42,71-73), and endothelial cells obtained from various animal tissues (74,75) and human umbilical cords (75,76). Mesangial cells occupy a central position in the renal glomerulus (77). These cells have characteristics of a modified smooth muscle cell, and are capable of a number of non-muscle functions such as the release of prostaglandins and other mediators of inflammation (77). Besides releasing potent vasoactive substances, mesangial cells can also contract or relax in response to several vasoactive substances released from neighboring mesangial cells or other cell types including platelets and endothelial cells (77). Thus, mesangial cells have the potential to be involved in acute functional CsA toxicity in two ways. First, the release of potent vasoconstrictors such as endothelin and thromboxane A_2 by mesangial cells can act on neighboring renal cells. Secondly, mesangial cells can contract in response to vasoactive peptides produced by other mesangial cells (autocrine) or cell types. This would ultimately result in increased renal vascular resistance and a subsequent decrease in renal blood flow, resulting in a decrease in GFR. Mesangial cells therefore appear to have an important role in the development and progression of CsA nephrotoxicity.

C. MECHANISM OF ACTION

The immunosuppressive effects of cyclosporine result from its action on T lymphocytes (3,21,76). Unlike conventional drugs commonly used in transplantation, it is neither cytotoxic nor myelosuppressive (79). Borel initially demonstrated that CsA reversibly inhibits selective T cell-mediated immune responses (7). CsA inhibits the activation cascade necessary for specific immune functions, particularly lymphokine production (78,80-83). The synthesis of interleukin-2, a lymphokine which promotes expansion of clones of effector lymphocytes activated by transplant antigens, is reduced by a single exposure to CsA both in vitro and in vivo in allograft recipients (78,81,83). Similarly, the drug also inhibits the synthesis of γ-interferon, which is an amplification signal for macrophages and monocytes (83) and other lymphokines including macrophage inhibitory factor and macrophage chemotactic factor (81,83).

The precise mechanism of inhibition by cyclosporine is not understood due in part to the limited knowledge of the exact activation pathways in T lymphocytes. However, in 1984, it was found that a cytoplasmic protein exhibited high binding affinity for CsA (84). This protein, termed cyclophilin, was first isolated from bovine thymus and later human spleen (85,86). The complete amino acid sequence of this protein was determined and found to lack any sequence homology to any protein thus far identified (85-87). Cyclophilin is an ubiquitous protein found in mammalian tissues, bacteria, and fungi (87).

Subsequent studies on cyclophilin have yielded much insight into its role in the mechanism of action of CsA. Studies by Harding et al (87) demonstrated that active derivatives of CsA have a much higher binding affinity to cyclophilin than do non-immunosuppressive analogues such as CsH. In 1989, it was demonstrated that cyclophilin had cis-trans peptidyl prolyl isomerase (PPIase) activity and was found to be homologous to PPIase isolated from bovine kidney (88,89). This enzyme is important in catalyzing the slow steps of protein folding. These studies have demonstrated that cyclosporine can specifically inhibit this activity, thus implying that cyclophilin has a pivotal role in the transduction of cell signals involved in immunosuppression (90).

The discovery that cyclophilin functions as an isomerase raises the possibility that recognition or isomerization of proline-containing epitopes may be relevant to the regulation of intracellular signalling events in T cell activation (90). Current evidence suggests that cyclophilin binds to a component of the transcriptional apparatus and thereby controls the activity of some yet unknown transcription factor(s). Since CsA specifically inhibits the function of certain nuclear proteins such as NF-AT and AP-3 (90), the refolding of these proteins may be instrumental for DNA binding or transcriptional activation.

D. CYCLOSPORINE STRUCTURE

Cyclosporine is a neutral, hydrophobic cyclic peptide composed of eleven amino acids with a molecular weight of 1202 Da (91-95). The

chemical structure of CsA is shown in Figure 1. There are a number of unique features of the peptide. The peptide contains D-alanine at position 8, an enantiomer not normally found in mammalian proteins. Seven of the amino acids are N-methylated (position 1,3,4,6,9,10,11) and all are aliphatic, giving rise to its highly lipophilic properties. The amino acid at position 1 of the peptide is unique and has never been previously isolated or identified in free form. This amino acid, N-methyl-4-[2-butenyl]-4-methyl threonine or MeBmt, is required, but not by itself sufficient, for full immunosuppressive activity (92,96). Amino acid residues 1, 2, 3, and 11 are required for full immunosuppressive activity (91). Alterations at these positions can be expected to diminish immunosuppressive activity. Interestingly, these amino acids are also required for binding to cyclophilin (92,93).

The chemical structure of CsA was established by chemical degradation studies (92,93,97) and its three-dimensional structure was determined by X-ray crystallography and nuclear magnetic resonance (NMR) studies (92,93,96,97). These studies revealed insight into the structure of CsA, which was found to be composed of two parts: an antiparallel β-pleated sheet (residues 1-6) stabilized by internal hydrogen bonding, and an open loop structure (residues 7-11). The anti-parallel β-pleated sheet contains a type II β-turn consisting of amino acids 2-5.

E. CYCLOSPORINE PHARMACOKINETICS

The pharmacokinetics of CsA have been extensively studied in both

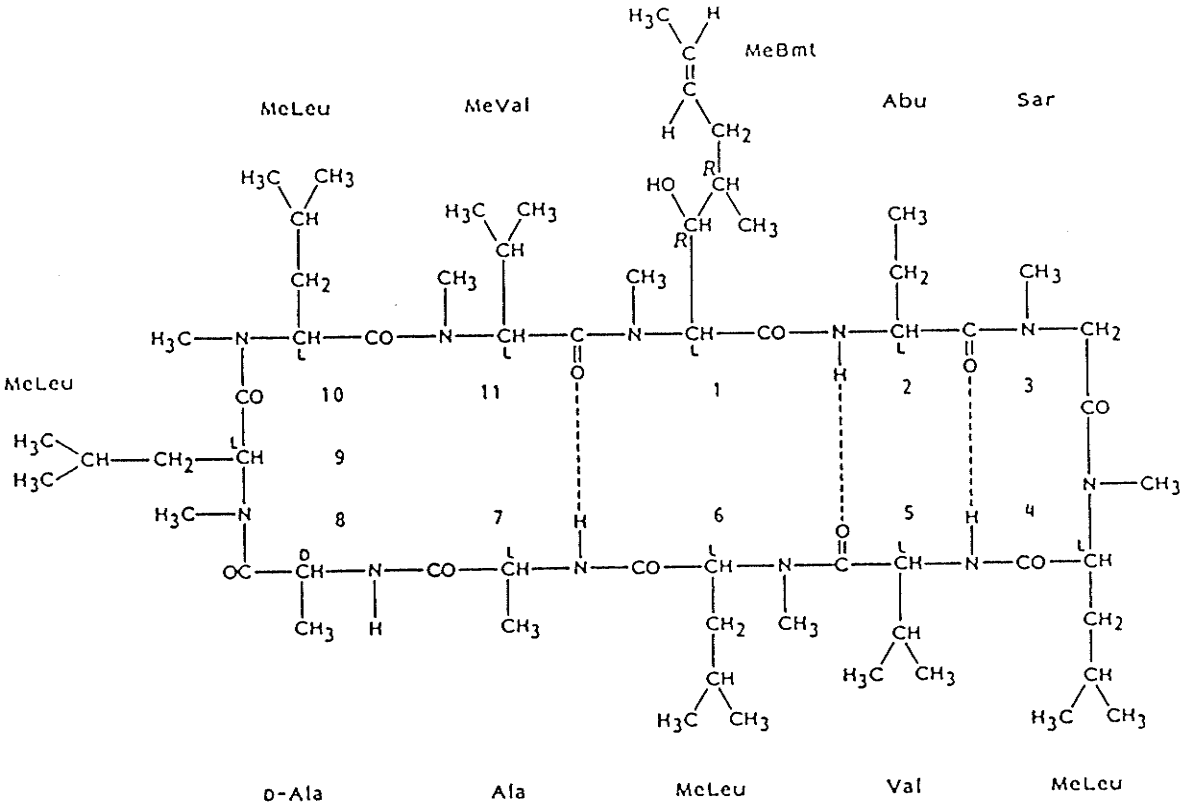

Figure 1. Chemical structure of cyclosporine.

animals and humans. CsA is absorbed by the upper part of the small intestine (99). Absorption is slow, variable, and incomplete and is influenced by a number of factors including the type of transplant, time after transplantation, functional status of the liver, presence of food in the stomach, other drugs, and intestinal dysfunction (13,95,99-103). Absorption of orally-administered CsA is slow, with peak blood levels being reached within 3 to 4 hours; however, the inter-patient variation is large (99). In renal patients, the time to maximum blood concentration has ranged from 1 to 8 hours (13,103). Absorption appears to be dependent on bile flow but the precise mechanism of absorption is not known. Gastrointestinal motility also affects absorption, with a moderate increase in the rate of gastric emptying drastically increasing the bioavailability of CsA (99-102). Bioavailability increases with time after transplantation, with values up to 57% being reported; however, in the postoperative period it is approximately 10% (13,99-103). Intravenously-injected CsA exhibits a multicompartment behavior (104). The drug has been found to exhibit two phases of distribution in humans (13). This phenomenon is attributed to the lipid solubility of CsA and its ability to diffuse through biological membranes.

CsA is widely distributed throughout the body and blood. The majority of CsA in whole blood is bound to erythrocytes with only 30 to 40% of the drug being found in the plasma (95,99,105). Most of the cyclosporine in the plasma is associated with lipoproteins (99,106); however, a significant amount is found bound to plasma proteins and lymphocytes. Less than 2% of cyclosporine circulates unbound in

plasma (106,109). CsA has a high volume of distribution because of its lipid solubility, extensive tissue binding, and loose binding to plasma proteins (99-103). The volume of distribution (Vd) represents the size of the compartment required to account for the total amount of drug in the body, if it were present in the same concentration as in the plasma. Estimates of the volume of distribution range from 3.5 to 9 L/kg (13,95,99-103). The large variation may be related to differences in binding of CsA to lipoproteins and erythrocytes in different patient populations.

CsA is readily sequestered by the liver. This may be related to the role of this organ in drug metabolism and excretion. In keeping with the lipophilic nature of CsA, the drug accumulates in fat (105,107,108). Highly vascularized organs such as heart, lung, and kidney have intermediate concentrations. Very low CsA concentrations are found in the brain and cerebrospinal fluid suggesting that CsA does not readily penetrate the blood-brain barrier (99,107,108). The drug persists in body tissues for a considerable time after CsA treatment is discontinued (13,95,99).

Cyclosporine is eliminated primarily by the liver in all species studied, including humans, rats, rabbits, and dogs (110,111). The clearance is a function of the ability of the liver to metabolize the drug, liver blood flow, and binding by blood proteins. Clearance of CsA varies markedly among patients and is influenced by the nature of the transplant, patient age, concurrent drug therapy, and disease state. The elimination half-life ($T_{\frac{1}{2}}$) of CsA ranges from 6.4 to 8.7 hours (13). There is appreciable variation in the clearance rate

between patients and transplant types. Drug clearance is 40% higher in children, and they therefore require larger doses (13,112). In contrast, elderly patients have a decreased clearance rate (95) and require less drug to maintain therapeutic blood levels. Numerous factors interfere with CsA elimination (110). Hepatic impairment reduces metabolite elimination. Numerous drugs interfere with the metabolism of CsA, both inducing and inhibiting its metabolism (113). The concurrent use of such drugs can drastically alter the pharmacokinetics of CsA (95,113). Drugs that induce P-450 enzyme activity, such as rifampin, phenytoin, phenobarbital, and carbamazepine, accelerate CsA metabolism and reduce CsA blood levels. In contrast, drugs such as erythromycin (114) and ketoconazole (115) lead to increased blood levels. Ketoconazole reduces P-450 activity, whereas erythromycin competes with CsA for binding sites on the enzyme. Erythromycin binds firmly to the P-450 complex forming an inactive complex (113).

F. CYCLOSPORINE METABOLISM

CsA is extensively metabolized by the liver in humans and animals by the cytochrome P-450 system (105,110,116,116-120). Two isoenzymes have been shown to be responsible. First, the P-450 IIIa, or the P-450 NF, which is responsible for the metabolism of many hydrophobic compounds including nifedipine, cortisol, erythromycin, and quinidine (117-119). A second cytochrome, P-450 hpCN3, has also been found to be involved in CsA metabolism (120). Cyclosporine and its metabolites are predominantly eliminated through the bile although a small but

significant amount is excreted in the urine (110,116). More than 90% of the administered intravenous dose is excreted as metabolites in bile. Subsequent to oral administration, only 6% of the dose is found in the urine with only 0.1% being excreted as the parent drug (105). In all metabolites identified to date, the cyclic structure has been preserved. The reactions involved in the biotransformation are limited to N-demethylation, hydroxylation, oxidation, and cyclization (110,116,121-127). N-demethylation appears to occur only at the N-methylleucine residue found at amino acid 4. Hydroxylation is restricted to the η-position of amino acid 1 and the γ-position of N-methylleucine at amino acids 4, 6, and 9. Intramolecular ether formation has been observed at amino acid 1.

Cyclosporine metabolites have been isolated from the bile or urine from a number of animal species as well as man using a number of different HPLC procedures (110,116,121-137). To date more than 20 metabolites have been isolated and characterized. The present nomenclature system used for CsA metabolites is based in part on their HPLC retention times (121). This has resulted in a lack of a systematic approach for naming newly isolated metabolites. It has recently been proposed that the nomenclature be revised and standardized to include information on their chemical structure (121,138,139). Table 1 lists the structural modifications of the major CsA metabolites which have been characterized to date. Both the present and proposed nomenclature system is included; however, in this thesis only the present system will be used. Several major metabolites of CsA have been identified by various investigators

Table 1. Structure of characterized CsA metabolites.

Metabolite nomenclature							
New[a]	Old	AA-1[b]	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	M-18	OH	CH_3	H	H	H	AA-1: Cyclization
AM1DI	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	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	M-203-218	COOH	CH_3	H	H	H	
AM1S	--	SO_4	CH_3	H	H	H	AA-1: Sulfation at β-Carbon

[a] Proposed at Hawk's Cay Meeting (139).

[b] AA-1: Amino Acid 1.

(95,110,116,121-137). Metabolites M-1 and M-17 are monohydroxylated derivatives of CsA, whereas M-8, M-10, and M-16 are dihydroxylated products of CsA. M-21 is a N-demethylated metabolite; metabolites M-13 and M-25 are also N-demethylated but are also monohydroxylated derivatives. M-18 results from intramolecular cyclization of M-17 and similarly M-26 is thought to result from a similar intramolecular cyclization of M-8. Definitive structural determinations of several other isolated metabolites have yet to be performed. This is partly due to the difficulty of isolating sufficient quantities of metabolites for subsequent structural determination. The proposed biotransformation pathways of CsA (95,110,116) suggest that metabolites M-17, M-1, and M-21 should be considered as primary metabolites, with other metabolites arising from their subsequent metabolism. This scheme, as shown in Figure 2, is based primarily on chemical structure rather than direct proof (13,95,110,116).

There have been several novel CsA metabolites which have been isolated and identified. A primary biliary metabolite of CsA has been isolated from both rabbit and human bile (125,126). The metabolite was identified as an acidic metabolite of CsA in which the η-CH_3 group of amino acid 1 has been oxidized to an α,β-unsaturated carboxylic acid (M-203-218). The formation of such a metabolite could be potentially important since aldehydes are highly reactive towards proteins. A novel, previously unidentified metabolite (M-E) has been identified in the blood of renal transplant patients (134). The structure of this metabolite is consistent with hydroxylation at an undetermined site. This metabolite was found to possess significant

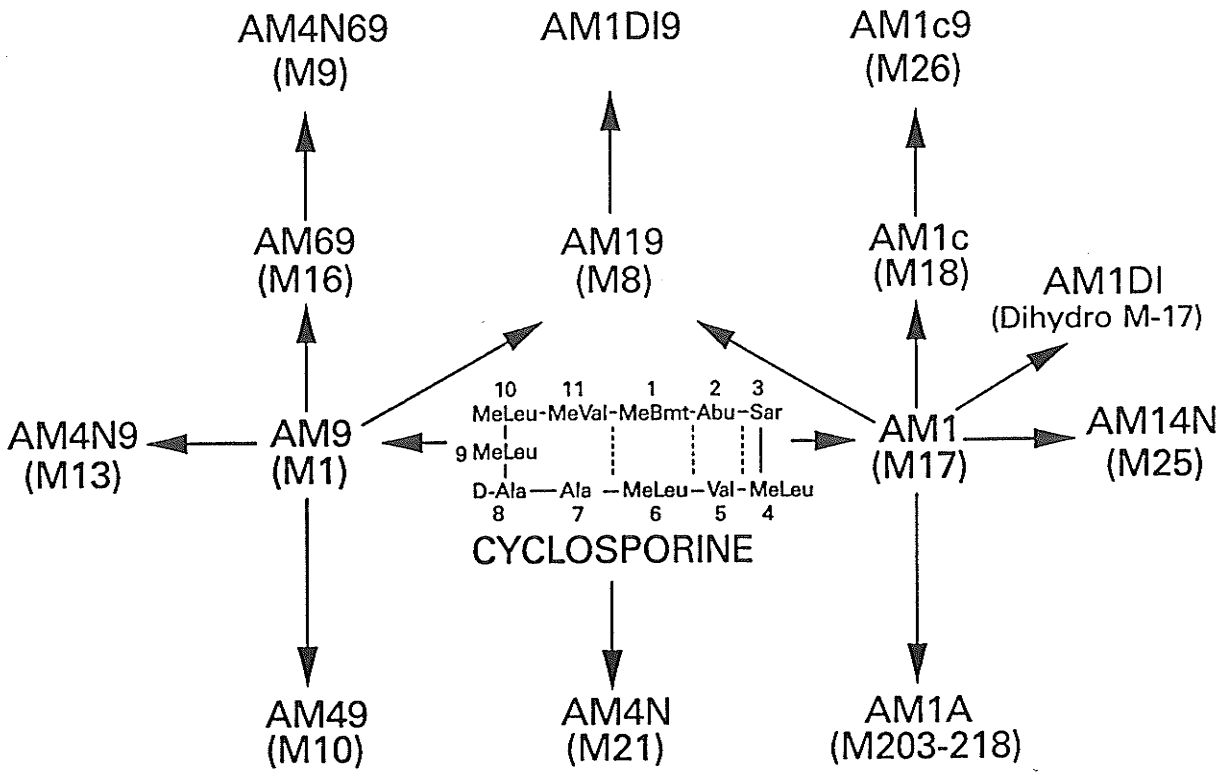

Figure 2. Proposed biotransformation of CsA and its metabolites.

immunosuppressive activity, being 79% as effective as CsA. A sulphate conjugate of CsA has also been isolated from human bile and plasma (136,137). All metabolites thus previously identified have been oxidized in nature; this is the first metabolite isolated that has been shown to be conjugated. For CsA, the only position that is available for conjugation is the hydroxyl group found in amino acid 1. The concentration of this metabolite in plasma is considerably higher than that of the parent drug (137). The conjugate, unlike other metabolites, seems to form ionized complexes, thereby increasing its water solubility.

Subsequent to an oral dose of CsA, the 24 hour (plasma) area under the curve (AUC) of the parent drug represents 53% of the total obtained for CsA and metabolites (105). Metabolites M-1, M-8, M-10, M-17, M-18, and M-21 were detected in plasma. Metabolite M-17 accounted for 13% of the total AUC, while the remaining metabolites ranged from 3 to 7% of the total. In erythrocytes, CsA and M-17 are the major components representing 27.4 and 24.4% of the AUC, respectively, whereas M-1 accounts for a further 13.8%. Trough blood concentrations of M-17 in specimens obtained from 24 renal transplant recipients ranged from 20 to 612 µg/L whereas CsA levels ranged from less than 20 to 310 µg/L (132). In a separate study in which CsA and metabolite concentrations were measured in 26 renal allograft recipients, the concentration of M-17 was usually greater than that obtained for CsA (140). It has been shown that the remaining metabolites are present in blood in concentrations less than CsA, with their relative concentration falling in the following order: M-1 =

M-8 > M-21 > M-203-218 > M-18 (140).

Tissue concentration of certain CsA metabolites has also been measured in humans and animals (105,110,116,122,141). The highest concentration of metabolites and parent drug are found in the kidney, liver, lymph nodes, fat, and skin (105,110,116,122,131). In renal cortical and medullary tissue obtained from human renal transplant recipients at time of nephrectomy, the level of M-17 was 3.5 to 4 times the level of CsA (131).

In studies with human kidney tissue obtained from CsA-treated patients, a high concentration of M-17 was determined along with lesser concentrations of CsA, M-1, and M-21 (131). The relative CsA and metabolite concentration in renal tissue paralleled the pattern in blood. Postmortem examination of adipose, kidney, liver, muscle, pancreas, spleen, and lung has revealed up to 53 fold higher concentrations of CsA and metabolites in tissue as compared to blood (142). The biological importance of metabolite distribution into tissues has not been established. Metabolite binding to cyclophilin has, however, been reported and an association between cyclophilin binding and immunosuppressive activity has been proposed (143).

The actual distribution of metabolites into potential target tissues may be important in assessing biological activity. Cellular distribution was first evaluated in blood where metabolites partitioned between plasma and blood cells. Initial HPLC studies revealed a greater cellular binding of M-17 and M-1 as compared to CsA and M-21 (131). A more recent study (122,130) 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, the hydroxylated metabolites (M-1, M-8, M-9, M-10, M-16, and M-17) preferentially partition into the cellular fraction while demethylated and cyclized metabolites (M-13, M-18, M-21, M-25, M-26, and M-203-218) remain primarily in the plasma. Blood distribution studies indicate significant cellular binding of metabolites (95,121). Because of the variability in partitioning, whole blood is required to clinically monitor CsA metabolite concentrations (95,121,138).

G. IMMUNOSUPPRESSIVE PROPERTIES OF CYCLOSPORINE METABOLITES

The immunosuppressive activity of CsA metabolites has been studied by a number of investigators using a variety of in vitro test systems (121,131,144-151). A summary of these studies is shown in Table 2. Rosano et al (131,144,145) initially examined the in vitro immunosuppressive activity of M-17, M-1, M-8, and M-21 using a mitogen stimulated [phytohemagglutinin (PHA) and concanavalin A (ConA)] and a mixed lymphocyte reaction (MLR) system. They demonstrated that M-17 exhibited a considerable amount of immunosuppressive activity in vitro, although it was not as inhibitory as CsA. M-21 and M-1 exhibited lesser activity and M-8 demonstrated no inhibitory activity. Further studies (144) in which they examined interleukin-2 production in addition to the previous systems, demonstrated that the order of inhibitory activity was CsA > M-17 > M-1 > M-21 > M-8. In the PHA assay used, CsA was significantly more inhibitory than M-17; however, in a ConA and mixed lymphocyte culture (MLC) system, the inhibitory

Table 2. In vitro immunosuppressive activity of CsA metabolites - study summary.

Investigator	Reference	Assay
Rosano (1986)	131	MLR, PHA, ConA, PWM
Rosano (1987)	144, 145	MLR-IL2, PHA, ConA, CTL
Wonigeit (1987)	149	MLR, CD3
Abecassis (1988)	146	ConA, MLR
Ryffel (1988)	150	MLR, PHA, ConA, CD3
Zeevi (1988)	147, 148	MLR, PLT, ConA

MLR: Mixed lymphocyte reaction.
PHA, ConA, PWM: Mitogen stimulated assays.
CTL: Cytotoxic T cell assay.
CD3: Anti CD3 stimulation.
PLT: Primed lymphocyte test.

activity of M-17 approached that of CsA. More importantly, M-17 and M-1 inhibited the production of IL-2 in the MLC to the same extent as CsA. In contrast, M-21 was significantly less inhibitory than either M-17 or M-1, and M-8 appeared to be devoid of biological activity. Abecassis et al (146) examined the immunosuppressive effects of cyclosporine metabolite fractions obtained from bile from liver transplant patients. Using a ConA and an MLR system, it was demonstrated that the CsA metabolites tested exhibited considerable immunosuppressive effects. The fraction containing M-17 was 60% as immunosuppressive as CsA in the MLR. This fraction in a ConA mitogen stimulated system was only 16% as effective as CsA. It should be noted, however, that this study (146) did not employ pure individual metabolites; the authors state that some of the metabolite fractions contained unidentified contaminants.

In addition to an MLR, Zeevi et al (147,148) used alloreactive T cells generated as clones from mixed lymphocyte cultures in a primed lymphocyte test to examine the effect of CsA and metabolites on its inhibition. None of the metabolites tested were as effective as CsA. M-17 followed by M-1 and M-21 exhibited the most significant in vitro immunosuppressive effects. This was particularly apparent in ConA and primary MLR stimulation assays, in which CsA was found to be about 100 fold more inhibitory than M-17, the most active metabolite. On the other hand, only 10 fold differences between CsA and M-17 were observed in the inhibition of the primed lymphocytes. Studies by Schlitt et al (149) examined the effect of M-17, M-1, and M-21 on the inhibition of T cell stimulation by an anti-CD3 monoclonal antibody

and in a MLR. None of the metabolites tested in either system were as immunosuppressive as CsA. M-17, which was about 10% as active as CsA, was the most immunosuppressive. Similar studies by Ryffel et al (150) demonstrated that CsA metabolites were 4 to 10 fold times less active than the parent compound on human lymphocyte activation using a variety of stimuli. M-17 was the most active metabolite tested, again being about 10% as active as CsA.

The immunosuppressive effects of CsA metabolites in vitro have differed between studies (see Table 2). This may be due to a number of factors (121). The purity of the metabolite used as well as the variable response of the culture techniques in some of these studies is suspect (144-150). In many cases, the data were not represented as a potency ratio or an IC_{50} with respect to CsA. This prevents an accurate comparison of the immunosuppressive activity of the metabolite in question to that of CsA. Assessment of purity was at best based on the metabolite eluting as a single symmetrical peak by HPLC. In many cases the metabolite examined was not free of contaminants. For example, Zeevi et al (147) state that their M-17 peak contained 10% unidentified material. The studies by Abecassis (146) examined metabolite fractions, some of which contained three identified metabolites. Furthermore, identification of metabolites was based on comparison of the retention time of the isolated metabolite to that of standard drug preparations obtained from the drug manufacturer.

H. TOXICITY OF CYCLOSPORINE METABOLITES

Studies on the in vitro renal toxicity of CsA metabolites have used both established and primary cell cultures. The porcine proximal tubule renal epithelial cell line ($LLC\text{-}PK_1$) has been extensively used to study CsA toxicity in vitro (63-69), as previously discussed. Becker et al (64) initially described the effects of CsA on this cell line. In subsequent studies, Cole et al (151) investigated the effect of two primary CsA metabolites, M-17 and M-1, on the growth of this cell line as well as a primary rat mesangial cell line. These metabolites at a concentration of 10,000 μg/L exerted minimal effect on DNA, RNA, or protein synthesis, while in contrast CsA at 500 μg/L resulted in significant inhibition of these metabolic functions in both cell lines.

Wilson and Hreniuk (152) 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 tubules (PCT) and proximal straight tubules (PST) were sensitive to CsA.

Studies investigating the effect of CsA metabolites on hepatocytes have also been reported (153-156). Boelsterli et al (153) demonstrated in a primary rat hepatocyte culture that CsA can decrease bile acid conjugation, and at very high concentrations (>1,000 μg/L) it can decrease protein synthesis. M-17 exhibited no toxic effects in this study. Further studies by this group investigated the

relationship between the extent of CsA metabolism and CsA-induced hepatotoxicity both in vivo and in primary rat hepatocytes (154). CsA administered (50 mg/kg/d p.o.) for 10 days in male Wistar rats demonstrated hepatotoxicity as measured by hyperbilirubinemia and increased bile salts. Treatment with Aroclor 1254 resulted in a 3.7 fold induction of CsA metabolism. This failed to change the degree of CsA-induced hepatotoxicity. Similar induction studies in vitro using dexamethasone (an inducer of the P-450 IIIc gene family responsible for formation of the primary CsA metabolites) failed to alter the degree of hepatotoxicity as measured by inhibition of protein synthesis. These results suggest that increased CsA biotransformation is not associated with a decrease in both in vitro and in vivo hepatotoxicity, suggesting that CsA metabolites may have an important role in CsA hepatotoxicity (154). Bowers (155) examined the effects of CsA metabolites on cultured rat hepatocytes. M-17 exhibited significant toxic effects as measured by inhibition of taurocholate conjugation and secretion, whereas M-18 and M-1 were less toxic. All metabolites were present at 1,000 μg/L. Riegel et al (156) 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 using this system.

Preliminary studies investigating the nephrotoxic properties of CsA metabolites in vivo have been reported (150,157-159). M-17, 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 (150). In contrast, a similar dose of CsA resulted in a significant

decrease in creatinine clearance and renal morphological changes consistent with CsA nephrotoxicity. Although the doses of CsA and M-17 were similar, the average concentration of M-17 in whole blood was less than half of that for CsA. It has recently been shown that M-17, M-18, M-21, and M-203-218 do not produce immunosuppression or nephrotoxicity when administered subcutaneously to rats for 10 days at 50 mg/kg/d (157). However, the results of this study were clouded by the fact that the AUC for the metabolites, in particular M-17, was significantly less than CsA. The lack of activity of metabolites may be due in part to low circulating 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 rat 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 M-1 as the major metabolite (160). In contrast, M-17 is the major blood and tissue metabolite in rabbits (111) and in renal transplant patients (130,142,160). Furthermore, in humans the AUC for metabolites often exceeds that of CsA (159,160). 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 M-17 have shown a rapid renal clearance of M-17 associated with low blood concentrations of the metabolite (158). 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 (159,160).

I. CLINICAL MONITORING OF CYCLOSPORINE

Clinical experience has shown that the therapeutic index for CsA is narrow (95). As previously discussed, both absorption and clearance of CsA varies widely (95,100-102). Because of this, the range of steady state concentrations of the drug presented to target-tissue receptors for a given dose is large. Therefore, monitoring of CsA concentrations in blood, serum, or plasma, in conjunction with other laboratory and clinical parameters is regarded as an essential aid in adjusting dosage for optimal efficacy with minimal toxicity.

Historically, the therapeutic range for CsA has been difficult to establish, due in part to the variation in sample matrix and selectivity of the assays as well as the clinical criteria used for the diagnosis of rejection and toxicity. This wide variety in monitoring protocols has made comparison of intra-laboratory results hard to interpret. A report of the Task Force on Cyclosporine Monitoring in 1987 made a number of recommendations for improved standardized monitoring of CsA in transplant recipients (95). It was suggested that whole blood be the preferred matrix for measurement of CsA; the method for measurement should be specific; and should metabolites be shown to be clinically significant, specific assays for their measurement are recommended.

Two recent reports have addressed recent advances in the therapeutic monitoring of CsA (138,139,161). The ultimate goal of

these documents is to provide the type of practical information that could foster an improvement in the centre-to-centre consistency in the practice of cyclosporine therapeutic drug monitoring. The Hawk's Cay consensus report (139,161) addressed areas of controversy but did not make specific recommendations for the method of measurement or sample type which was addressed at the Canadian Consensus Meeting on Cyclosporine Monitoring (138).

Briefly, it was recommended that whole blood be the sample matrix of choice because of analytical reasons. A consistent sample matrix would make the comparison of inter-laboratory samples more meaningful. The specific measurement of CsA in which only parent drug and not the metabolites are quantified can be accomplished by two procedures: HPLC and immunoassays involving a selective monoclonal antibody. The former, when well validated, has been considered the reference method for the specific measurement of the drug. Several HPLC procedures have been described (121) with the major differences among them being sample preparation and chromatographic procedures. Regardless of the assay, the method should be validated and its performance characteristics should meet acceptable criteria.

The recommendations of these reports should help standardize CsA monitoring. Taking into account two recommendations proposed, namely the use of selective assays with standards and samples in whole blood matrix, should facilitate the establishment of a consensus therapeutic range. The therapeutic range varies among transplant centres and depends upon the transplant type and the time post-transplant. The typical range for renal transplant recipients receiving CsA orally

twice a day is approximately 150 to 300 μg/L immediately post-transplant with the range decreasing to 100 to 150 μg/L as the time post-transplant increases to three months. In general the therapeutic range for liver transplants is higher than that for renal recipients (250 to 350 μg/L <3 months; 100 to 250 μg/L >3 months).

There is general agreement that the frequency of nephrotoxicity and hepatotoxicity increases with increasing concentrations of the drug, while the chances of rejection increases as CsA concentration decreases (95). The incidence of CsA-induced structural alterations in the kidney is correlated to drug dosage (95,162,163). Major lesions are seen in patients having received high dosages (>7.5 mg/kg/d). The early renal dysfunction observed within 2 to 3 months is reversible by dosage reduction. For a given average blood level, there are wide variations in the extent of nephrotoxicity. Moyer et al (163) reported that CsA nephrotoxicity could be minimized by adjusting the dosage on the basis of drug concentration in blood. By maintaining trough whole blood concentrations in the range of 150 to 250 μg/L during the first 4 months (as measured by HPLC), toxicity was reduced to a minimum. Holt et al (164) studied CsA blood levels in renal transplant patients during episodes of renal allograft dysfunction in an attempt to assess whether a specific measurement of CsA (parent drug only) was more useful than a non-specific (parent drug plus metabolites) measurement. These results suggested that the measurement of CsA alone was a better guide to the differential diagnosis of renal allograft dysfunction than the previously used non-specific RIA (which demonstrated considerable cross-reactivity

with CsA metabolites). While there was a marked trend for nephrotoxic episodes to be associated with cyclosporine concentrations, the overlap between the concentration associated with rejection and nephrotoxicity was large. A prospective study by Lindholm et al (165) examined the relationship between CsA concentration to its therapeutic effect and toxicity in renal transplant recipients. Again, a significant relationship between CsA concentrations and clinical events was observed, with toxicity occurring at higher blood concentrations. However, when a polyclonal non-specific RIA method was used, there was no significant correlation, suggesting that the measurement of parent drug alone correlates better with the clinical course, be it toxicity or rejection.

Although adverse clinical events tend to correlate with trough levels, almost half of renal transplant recipients have drug levels that are inconsistent with their renal status, suggesting considerable overlap among patients in values for effective, ineffective, and toxic trough concentrations (13). A study by Sommer et al (166) failed to show a correlation between nephrotoxicity or rejection episodes following CsA treatment in renal allograft recipients. Furthermore, a study by Kumar et al (167) examined the effect of CsA dose on blood and tissue levels in rats administered the drug. A correlation was observed between the severity of the histological features and the CsA levels in the respective tissues. In contrast, no correlation was observed between the toxic effects and blood concentrations, thus suggesting that tissue levels may more reflect what is occurring than blood levels.

Clinical studies measuring both parent drug and metabolite concentrations have yielded only limited information concerning the concentration and potential clinical significance of CsA metabolites (121). High circulating levels of the primary metabolites in trough blood have been reported for most transplant types (122,130,131,140). In addition to M-17, other metabolites, including M-1, M-8, M-18, M-25, M-13, M-26, M-203-218, as well as other metabolites have been detected in the blood of CsA-treated patients (121,122,130,131,140). In contrast to rat studies that showed only a minor amount of metabolites in blood (110,160), the total concentration of metabolites from CsA-treated patients exceeds that of the parent drug (95,105,110). 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 metabolites (142,158). The correlation between the half-life ($T_{\frac{1}{2}}$) of CsA and that of M-17 and M-1 suggests that these metabolite are rate-limiting for parent drug elimination. Serial trough profiles of metabolites (140) as well as periodic pharmacokinetic studies (142) during the first four months post-transplant both showed no relative changes in the concentration of CsA and M-17. Clinical studies, therefore, indicate a relatively high concentration of metabolites in blood and suggest a significant exposure of peripheral tissues to these biotransformation products.

A significant correlation of metabolite concentration with clinical events would provide the strongest practical justification for monitoring specific metabolites in routine practice. High CsA

metabolite blood concentration has been associated with nephrotoxicity (140,169). However, clinical studies to test the correlation of specific metabolites with clinical events such as graft rejection and nephrotoxicity are limited both in number and conclusion. 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. The absolute and relative concentrations of CsA and metabolites did not differ significantly in the two groups of patients (95). In contrast, a study by Yee et al (170) examined the effect of CsA and metabolite levels on nephrotoxicity in bone marrow transplantation. CsA blood levels did not correlate with the nephrotoxic episodes; however, measurement of CsA and metabolites (as measured by a non-specific RIA) did correlate. This study suggested that CsA metabolites may have a significant role in the development of nephrotoxicity.

Studies on liver transplant recipients have suggested a role of CsA metabolites in nephrotoxicity. In contrast to kidney transplant recipients, nephrotoxicity in these patients can be differentially diagnosed from kidney rejection. Furthermore, these patients often show great variation in their CsA metabolite pattern. Wonigeit et al (171) measured CsA blood levels by non-specific and specific RIA methods. They showed that CsA nephrotoxicity in the early course after liver transplantation is associated with two distinct patterns of blood levels. One pattern is characterized by increased parent

drug levels, the other by an increased metabolite concentration resulting from an altered CsA metabolite excretion. This raises the possibility that not only the parent drug but also some of its metabolites may exert nephrotoxic effects when present in excessively high concentrations. Thus, this study provides the rationale for measuring CsA by both specific and non-specific measurements. Previous studies by this group (169) demonstrated an association of very high blood levels of CsA metabolites with clinical complications after liver transplantation, providing further evidence for the importance of CsA metabolites in the development of nephrotoxicity.

Sewing et al (172) studied the nephrotoxicity of CsA and its metabolites in a prospective study which included liver allograft recipients. They found a high correlation between nephrotoxicity and the double hydroxylated CsA metabolites, especially with M-26, but not with CsA itself. Whether this metabolite is directly involved in toxicity remains to be investigated. It is of interest that alteration of the CsA metabolite pattern during rejection resulted in an increase in concentration of M-8 and M-203-218, whereas other metabolite levels were not affected. Thus CsA monitoring, including the CsA metabolite pattern, is efficient in detecting and preventing CsA metabolite toxicity.

J. CYCLOSPORIN ANALOGUES

Recognition of CsA toxicity has lead to a search for CsA analogues that maintain immunosuppressive potency but exhibit less toxic effects. In addition to CsA, Tolypocladium inflatum produces a

large number of minor metabolites of the same structural type. At least 25 of these natural cyclosporines have been isolated and their chemical composition determined (94). In addition, over 750 semisynthetic analogues have been produced and tested in vitro, but only a few have been available in significant amounts for in vivo characterization. To date none of the natural or synthetic analogues have been shown to possess greater pharmacological potency than CsA in all test systems examined (94,98).

The first analogues to have been studied in detail were dihydro-cyclosporin C (CsC), in which threonine replaces α-aminobutyric acid at amino acid 2, and cyclosporin D (CsD), in which valine is found at amino acid 2. Dihydro-CsC was found to inhibit both cell-mediated and antibody-mediated immunity in contrast to CsA which inhibits only the former (94,173,174). CsD was shown to be much less immunosuppressive than CsA. CsD lacked nephrotoxic effects, but exhibited hepatotoxicity (173,174).

K. CYCLOSPORIN G (NVa^2-CYCLOSPORINE)

Of all CsA analogues examined, cyclosporin G (CsG) has been the most widely studied (94,98,175,176). It differs from CsA by substitution of L-norvaline for α-aminobutyric acid at the amino acid 2 position of the molecule. In vitro and in vivo studies involving mitogenic stimulation, mixed lymphocyte reactions, and local graft vs host reactions have shown CsG to be as immunosuppressive as CsA (177). CsG has been shown to be as potent as CsA in the inhibition of lymphokine production, including γ-interferon, lymphotoxin, and tumour

necrosis factor activity, as measured by mitogen and alloantigen stimulated peripheral blood mononuclear cells from normal individuals (178). The in vivo immunosuppressive activity of CsG has been compared to CsA using kidney and liver transplants in dogs (179,180), and hearts in non-human primates (181). In the majority of these studies CsG was found to be equipotent as CsA in the prevention of allograft rejection, although in some studies a lesser potency was observed (179-183). The reasons for these conflicting data are unclear. It could possibly be due to variations in the dosage and route of administration of the drug as well as species and organ transplantation differences from study to study (94,98). Currently, clinical studies are underway examining the use of CsG in the prevention of allograft rejection in human renal transplantation.

There have been several reports investigating the toxicity of CsG. In experiments using mice and rats, CsG was found to be less nephrotoxic than CsA when morphological and biochemical parameters were examined (179,180,184,185). Renal plasma flow and glomerular filtration rate were unaltered in CsG-treated rats compared to controls but significant alterations were observed in similarly treated animals receiving CsA (25 mg/kg/d for 21 days). Histologically, renal tubular toxicity characterized by vacuolization and microcalcification were seen in greater frequency among CsA as compared to CsG-treated animals. In other studies in the rat, canine and primate models, CsG has been shown to be more hepatotoxic than CsA as evidenced by increased serum levels of bilirubin and hepatic enzymes (179,184).

The pharmacokinetics of CsG are similar to CsA in both animal and clinical studies (174,177,186,187). Single dose pharmacokinetic studies have shown that the half-life and volume of distribution of CsG were not significantly different from CsA (187). The pharmacokinetics of CsA and CsG were investigated in 6 patients with terminal renal failure after a 4 hour intravenous infusion (3.5 mg/kg) and after oral administration (600 mg) of the drug (187). Based on HPLC determination of blood concentrations, the resulting pharmacokinetic parameters of CsG were similar to CsA. Subsequent to oral administration, maximum CsG concentrations in blood were reached between 2.5 and 3 hours (similar to CsA) and the bioavailability was 24 to 55% (187). It has been shown that the blood/plasma ratio of CsG is 1.23 which is smaller than that observed for CsA. This suggests that although whole blood levels of the drug would be similar, the plasma concentration would be higher than CsA. Furthermore, the temperature-dependent distribution of CsG differs from that of CsA in whole blood (188). It appears that CsG is more tightly bound to erythrocytes than CsA thus requiring longer incubation times for plasma-erythrocyte re-equilibration of the drug to occur (188).

To date, little is known about the metabolism of CsG. Since it is structurally similar to CsA, it is likely that it is metabolized by the same P-450 isoenzymes responsible for the metabolism of CsA (116-120), although this remains to be proven. Similarly, the role that CsG metabolites may have in overall immunosuppression and toxicity is not known. These areas need to be investigated in a manner previously done for CsA to determine whether parent drug alone

or in combination with metabolites should be monitored as a guide for dosage adjustments to maximize the drug's therapeutic effect while minimizing its toxic effects. The answer to these questions should aid in the therapeutic monitoring of CsG in clinical situations.

II. OBJECTIVES

The data available on the immunosuppressive and toxic properties of CsA metabolites are both limited and inconclusive. It is presently not known whether CsA alone or CsA and one or more of its metabolites should be monitored to aid in the adjustment of CsA administered so that optimal immunosuppression is obtained with minimal side effects. Metabolites may exist which are immunosuppressive but which lack many of the unwanted side effects, most importantly nephrotoxicity. If one or more CsA metabolite(s) are found to possess significant immunosuppressive activity, this would warrant its measurement to optimize CsA therapy. Nephrotoxicity is one of the major complications of CsA therapy. It is presently unclear whether CsA alone and/or one or more of its metabolites are nephrotoxic. Since CsA metabolites can be found in significant concentrations in tissue, often exceeding that of the parent drug, their potential role cannot be overlooked. The finding of CsA metabolites that are nephrotoxic may lead to the monitoring of their concentration along with CsA in blood as an approach to minimize toxicity. Because of the nephrotoxicity of CsA, there has been an active search for CsA analogues which are immunosuppressive but less nephrotoxic. In the clinical studies presently underway with CsG, it is not known whether only CsG or CsG plus one or more metabolites should be measured to allow appropriate dosage adjustments to be made to maximize immunosuppression while minimizing toxicity. The role of CsG metabolites in overall immunosuppression must be elucidated prior to

answering this question.

This thesis will address the role of two classes of cyclosporin analogues, namely metabolites of the parent drug, and a naturally occurring analogue, CsG, in immunosuppression and nephrotoxicity. In view of the above, the objectives of this thesis are:

1. To isolate CsA and CsG metabolites.

2. To structurally identify the isolated CsA and CsG metabolites using mass spectrometry (MS) and nuclear magnetic resonance (NMR).

3. To determine the immunosuppressive properties of the isolated CsA and CsG metabolites using a number of in vitro systems.

4. To investigate the pharmacokinetics and biotransformation of the CsA metabolite M-17 in the rabbit.

5. To investigate the cytotoxicity and functional toxicity of CsA metabolites in a primary and cultured renal cell line.

III. CHAPTER 1

ISOLATION AND CHARACTERIZATION OF CsA METABOLITES

METHODS

A. MEASUREMENT OF CsA IN WHOLE BLOOD BY HPLC

The concentration of CsA in whole blood was measured by HPLC using a modification of the procedure of Carruthers et al (189) and revised by our laboratory (190). Briefly, to 1 mL of whole blood, 25 µL of 20,000 µg/L internal standard was added [cyclosporin G (CsG)] followed by the addition of 2 mL of 0.2 N NaOH and 6 mL of diethyl ether. Subsequent to shaking for 10 minutes the ether layer was removed and 2 mL of 0.2 N HCl was added and shaken for 10 minutes. The diethyl ether layer was removed, evaporated under nitrogen, and reconstituted in 300 µL mobile phase [acetonitrile/methanol/water; 53/20/27 (v/v/v)]. Three hundred µL of hexane was added, the mixture vortexed for 30 seconds, and centrifuged at 800 xg for 5 minutes. The hexane layer was discarded and the mobile phase collected for subsequent analysis by HPLC.

Chromatographic separation of CsA was performed using a Varian HPLC (Varian Inc., Walnut Creek, CA, USA). The separation was performed isocratically using a spherasorb C-8 column (5 µm, 25 x 0.46 cm) (Chromatographic Specialties Inc., Rexdale, ON) preceded by a pellicular silica precolumn (Upchurch Scientific, Oak Harbour, WA, USA) using the mobile phase described above. The flow rate of the solvent was 1.0 mL/minute, the column temperature was maintained at

70°C, and the absorbance was measured at 214 nm. The concentration of CsA was determined by the ratio of the area under the curve of CsA to that of the internal standard. Since the molar absorption coefficient of CsA is slightly higher than that of CsG (internal standard), the ratio of the area under the curve of equal concentrations of CsA and CsG was determined at 214 nm. The CsA/CsG area ratio was generally 1.05. This factor was included in the final calculation of CsA concentrations.

B. ISOLATION OF CsA METABOLITES

CsA metabolites were isolated from two sources. First, CsA metabolites were isolated from urine obtained from renal transplant patients receiving CsA as part of their immunosuppressive therapy (Health Sciences Centre, Winnipeg, MB). Second, metabolites were isolated from human bile obtained from liver transplant recipients receiving CsA from Dr. D. Grant (Department of Surgery, University Hospital, London, ON) and Dr. Jacques Corman (Notre Dame Hospital, Montreal, PQ). CsA metabolites were extracted from urine using a modification of the procedure described above for whole blood (191,192). Briefly, to 40 to 50 mL of urine (pH adjusted to 10 with 2 N NaOH), 50 mL of HPLC grade diethyl ether was added. Subsequent to shaking for 10 minutes, the aqueous layer was removed. The above procedure was repeated three or more times using "fresh" urine and the same diethyl ether. Subsequently, 50 mL of 0.2 N HCl was added to the ether layer and shaken for 10 minutes. The diethyl ether layer was removed, evaporated under nitrogen, reconstituted in 500 µL mobile

phase [acetonitrile/methanol/water; 40/20/40 (v/v/v)], and washed with hexane as described above.

Chromatographic separation was similar to that described above for CsA quantification with the following modifications: 1) In some instances two C-8 columns in tandem were used. This resulted in enhanced chromatographic resolution. 2) In some instances the concentration of acetonitrile in the mobile phase was modified. Decreasing the acetonitrile concentration of the mobile phase increased its polarity. This resulted in increased retention times for the eluting metabolites. In most cases, this resulted in better chromatographic separation of two closely eluting peaks. This was particularly useful in achieving adequate separation of early eluting peaks. Fractions corresponding to the peaks of interest were collected manually and the mobile phase evaporated under low heat. Corresponding fractions from multiple injections were pooled for subsequent experiments.

The extraction of CsA metabolites from bile was similar to that described above for urine, except that bile was used in place of urine.

C. IDENTIFICATION AND CONFIRMATION OF PURITY OF ISOLATED CsA METABOLITES

The purity of the isolated CsA metabolites was initially assessed as the presence of a single peak by HPLC. Subsequently, structure and purity were determined by mass spectroscopy (MS) and nuclear magnetic resonance (NMR) performed by Dr. J. Westmore and Dr. T. Schaefer,

respectively (Department of Chemistry, University of Manitoba).

The molecular weight of the metabolites was determined by fast atom bombardment mass spectroscopy (FAB/MS) (191,192). Mass spectrometry was performed using a VG 7070E-HF mass spectrometer equipped with a fast atomic bombardment source, a post-acceleration detector and a 2505 data system (VG MassLab, Altracham, UK). The matrix used to solubilize the sample was dithiothreitol/dithioerythritol (5:1 v/v). Approximately 10 to 50 μg of metabolite was required for each analysis. High-purity research grade Xenon (99.99%) was used as the bombardment gas, and the resulting positive ions extracted into the mass analyzer. A mass range of 100 to 1500 daltons was scanned.

Proton-NMR and ^{13}C-NMR analysis of the CsA metabolites was performed at 300 MHz in $CDCl_3$ using a Bruker WH90/AAM300 NMR (Bruker Instruments Inc., Silberstreiten, FRG) with tetramethylsilane being used as an internal standard (191,192). About 100 μg of sample was required for proton-NMR; in contrast, greater than 1 mg was required for ^{13}C-NMR analysis. More material was required for ^{13}C-NMR analysis because of the relatively low natural abundance of this isotope. The ^{13}C isotope represents only 1.1% of all naturally occurring carbon atoms (193). In contrast, protons represent >99% of all naturally occurring hydrogen atoms (193).

D. DETERMINATION OF CsA METABOLITE CONCENTRATIONS

The concentration of CsA metabolites was measured using the HPLC procedure described above. The dried metabolite fraction was

reconstituted in 1 mL of methanol. An aliquot, usually 10 µL, was removed. To this, 50 µL of cyclosporin C (CsC) internal standard (50,000 µg/L) and 200 µL of mobile phase was added. The sample was chromatographed as previously described. The amount of metabolite was calculated by comparing the area under the curve of the metabolite to that of the internal standard. It was assumed that both metabolites and CsA had similar molar absorption coefficients at 214 nm. This assumption was based on the structural similarities of CsA with its metabolites. In a recent publication, Fu and Bowers (194) have determined the absorption coefficients for a number of CsA metabolites. On average, the molar absorptivity of the metabolites examined was 10% higher at 214 nm than that obtained for CsA.

E. CROSS-REACTIVITY STUDIES

Drug-free whole blood obtained from healthy volunteers was spiked with individual CsA metabolites to a final concentration between 500 and 5,000 µg/L. CsA metabolite concentrations were measured as CsA by radioimmunoassay (RIA) (Sandimmune RIA, Sandoz Inc., Basel, Switzerland) using either a monoclonal selective or monoclonal non-selective antibody, as previously described (190,195). The percentage cross-reactivity was determined using the following equation: (metabolite recovered as measured as CsA/metabolite concentration added) x 100. Each metabolite was analyzed in duplicate on three separate days.

RESULTS

A. MASS SPECTROMETRY AND NMR STUDIES OF CsA

The FAB/MS and proton-NMR spectrum of CsA is depicted in Figure 3. FAB/MS analysis of CsA produced a major parent ion of m/z 1203 (MH^+), which is a 1 Da increase in the mass of CsA (1201.64 Da). This protonated parent ion is a result of the FAB process. No other major ions were observed, however an ion of m/z 1090 (MH^+), a loss of 113 Da, was observed. This fragment was a result of the loss of the side chain of amino acid 1 (dimethyl butenyl threonine). The proton-NMR spectrum of CsA is depicted in the bottom of Figure 3. The protons of the free amino groups were represented as doublets with chemical shifts of 7.0 to 8.5 ppm, the α-carbons at shifts 4.0 to 6.0 ppm, and the methylated amino groups at shifts 2.5 to 3.5 ppm. A peak of particular interest corresponding to the η-CH_3 proton of amino acid 1 (AA-1) was observed at 1.62 ppm. The relevance of this peak will be discussed below. The ^{13}C-NMR spectrum of CsA is depicted in Figure 4. This technique gives reliable information as to the environment of all carbon atoms in the molecule. The ^{13}C-NMR chemical shifts for CsA have previously been assigned (97) and are listed in Table 4 (see later). This information will assist in the assignment of the ^{13}C-NMR chemical shifts for the isolated CsA metabolites.

B. IDENTIFICATION AND CHARACTERIZATION OF CsA METABOLITES

Figure 5 depicts a representative chromatogram of the separated CsA metabolites that were observed in the diethyl ether extract of

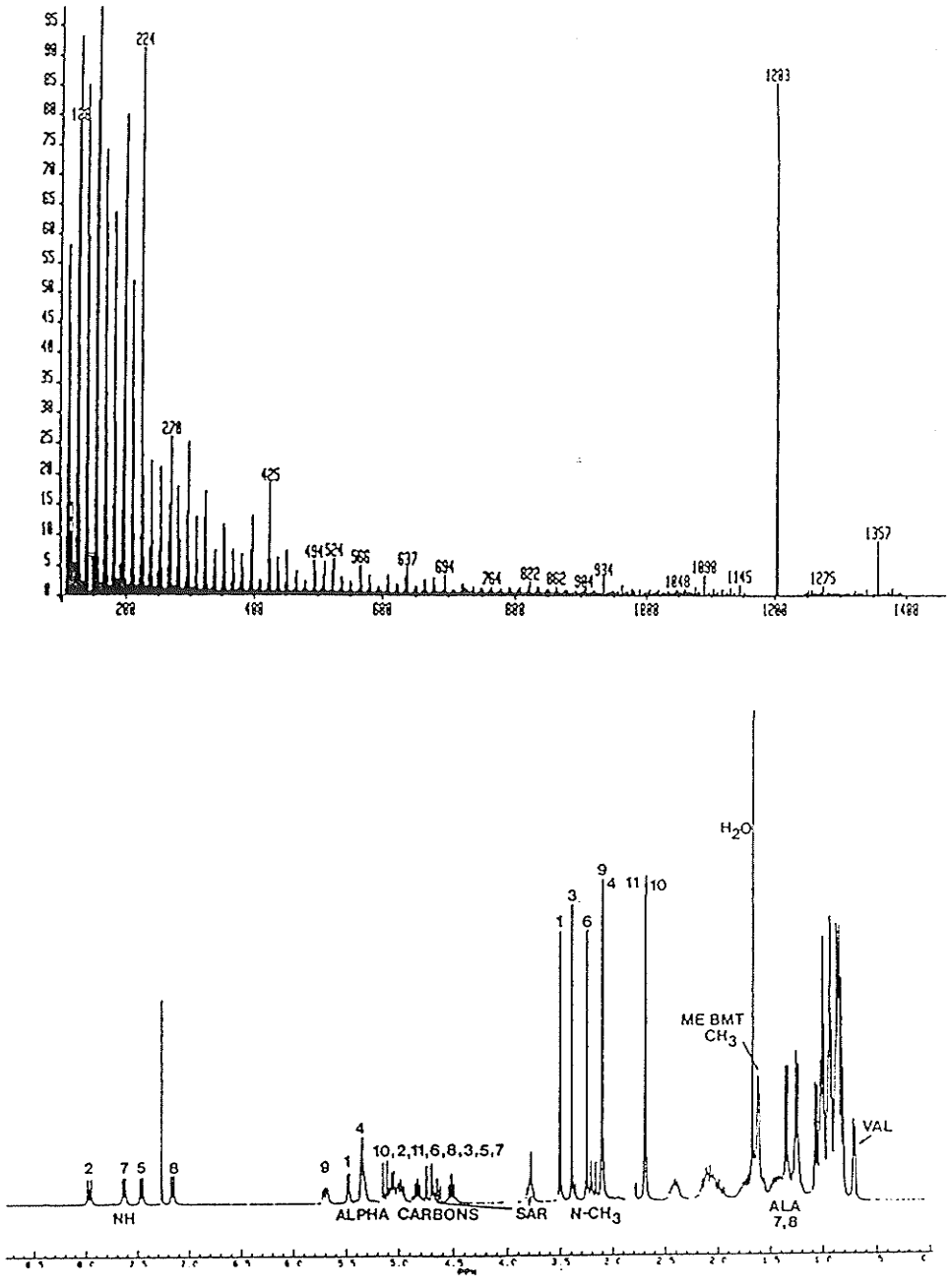

Figure 3. FAB/MS and proton-NMR spectra of CsA. FAB/MS analysis (Top) revealed a parent ion of MH^+ of 1203 Da. Proton-NMR (Bottom) is labelled with respect to the various protons of interest. The numbers above the spectra identify the amino acids which were identified.

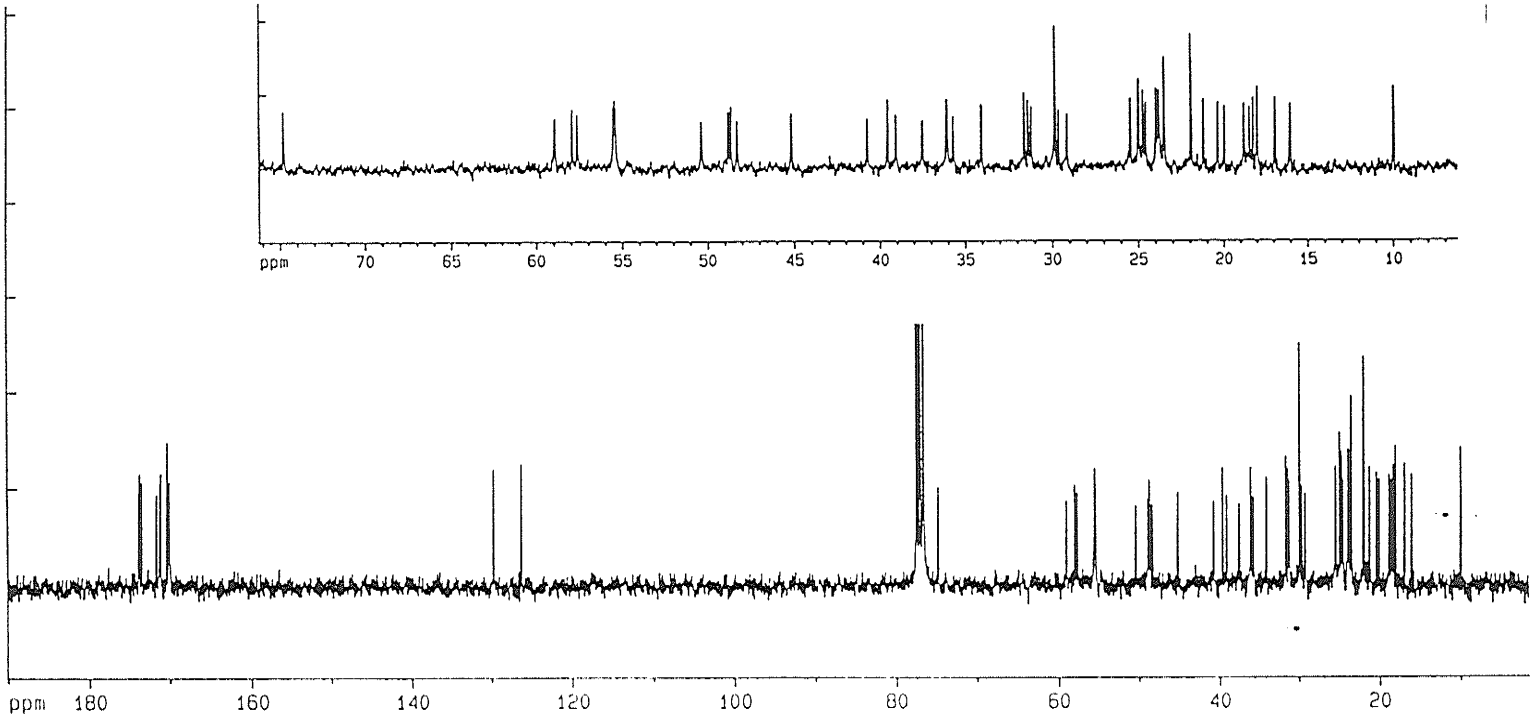

Figure 4. ^{13}C-NMR spectrum of CsA. The ^{13}C-NMR spectrum of CsA from 10 to 180 ppm is depicted. The insert represents an enlargement of the spectrum from 10 to 75 ppm.

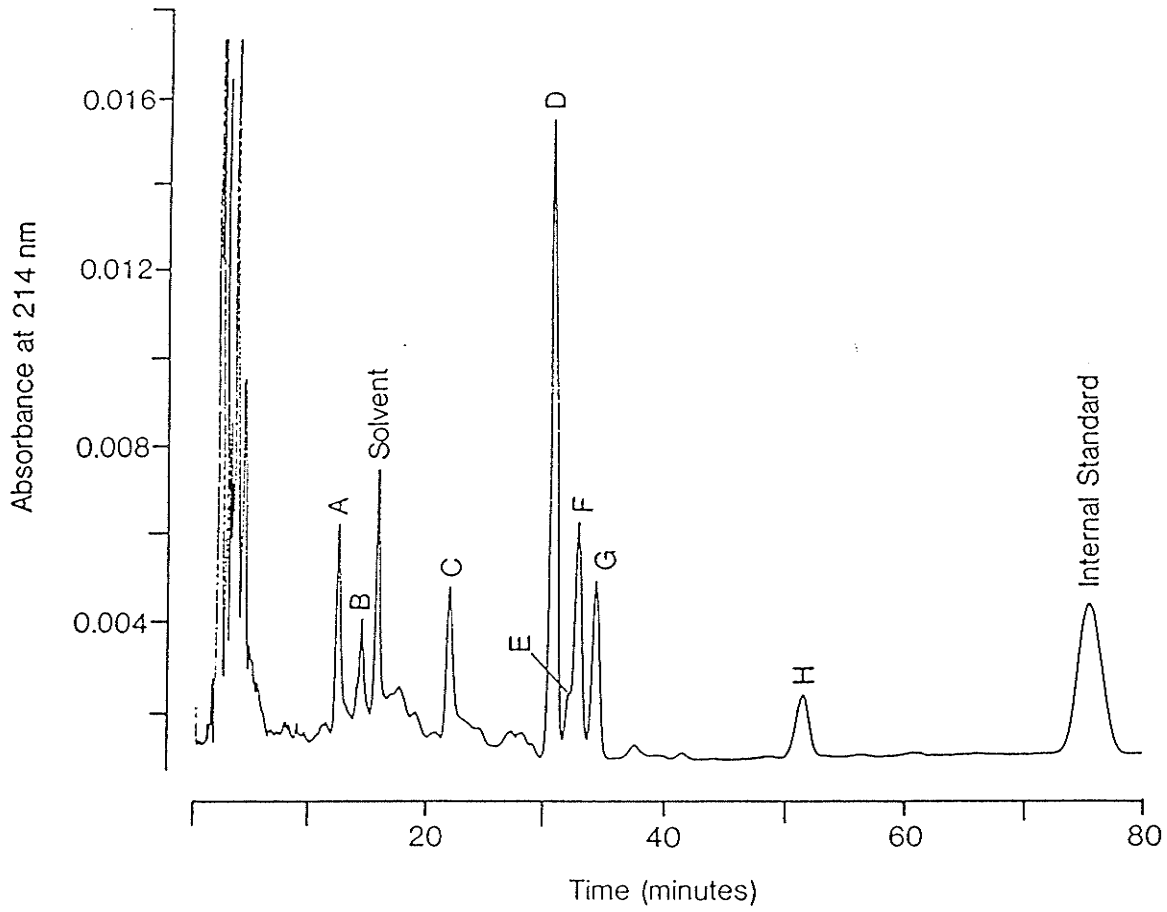

Figure 5. HPLC profile of CsA metabolites isolated from the urine of renal transplant recipients. Peaks A to H represent the individual metabolites isolated. The internal standard used was Cyclosporin C (CsC).

urine obtained from renal transplant recipients. By comparison, Figure 6 depicts the profile obtained from bile obtained from liver transplant recipients. There are a number of similarities between the spectra. The metabolite profile is similar for the two sources, however the relative proportions of individual metabolites differ. Bile appears to have a higher percentage of polar metabolites than that found in urine. This is evidenced by the increased amount of early eluting peaks with respect to the later eluting hydrophilic ones. The yield of metabolites was much higher from a litre of bile versus that obtained from a litre of urine (greater than 3 fold); however, because of the limited availability of bile, urine was used as a major source of metabolites.

The major peaks of the urine profile depicted in Figure 5 are labelled A through H. These peaks were collected from multiple injections in which the eluted fractions were pooled. Subsequently, the identity of the isolated metabolite peaks were determined as discussed below. Table 3 summarizes the structural information, including the site of modification, gained from FAB/MS, proton-NMR, and in most cases ^{13}C-NMR for the eight identified peaks. The structural assignment was derived using the rationale described below.

Peak A: The protonated molecular ion of this metabolite peak was observed in the FAB/MS at m/z 1235 (MH^+), corresponding to a 32 Da increase in molecular mass as compared with the protonated molecular ion of parent CsA (m/z 1203 MH^+). A fragment of m/z 1106 Da was observed, resulting from the loss of 129 Da from the parent ion, as

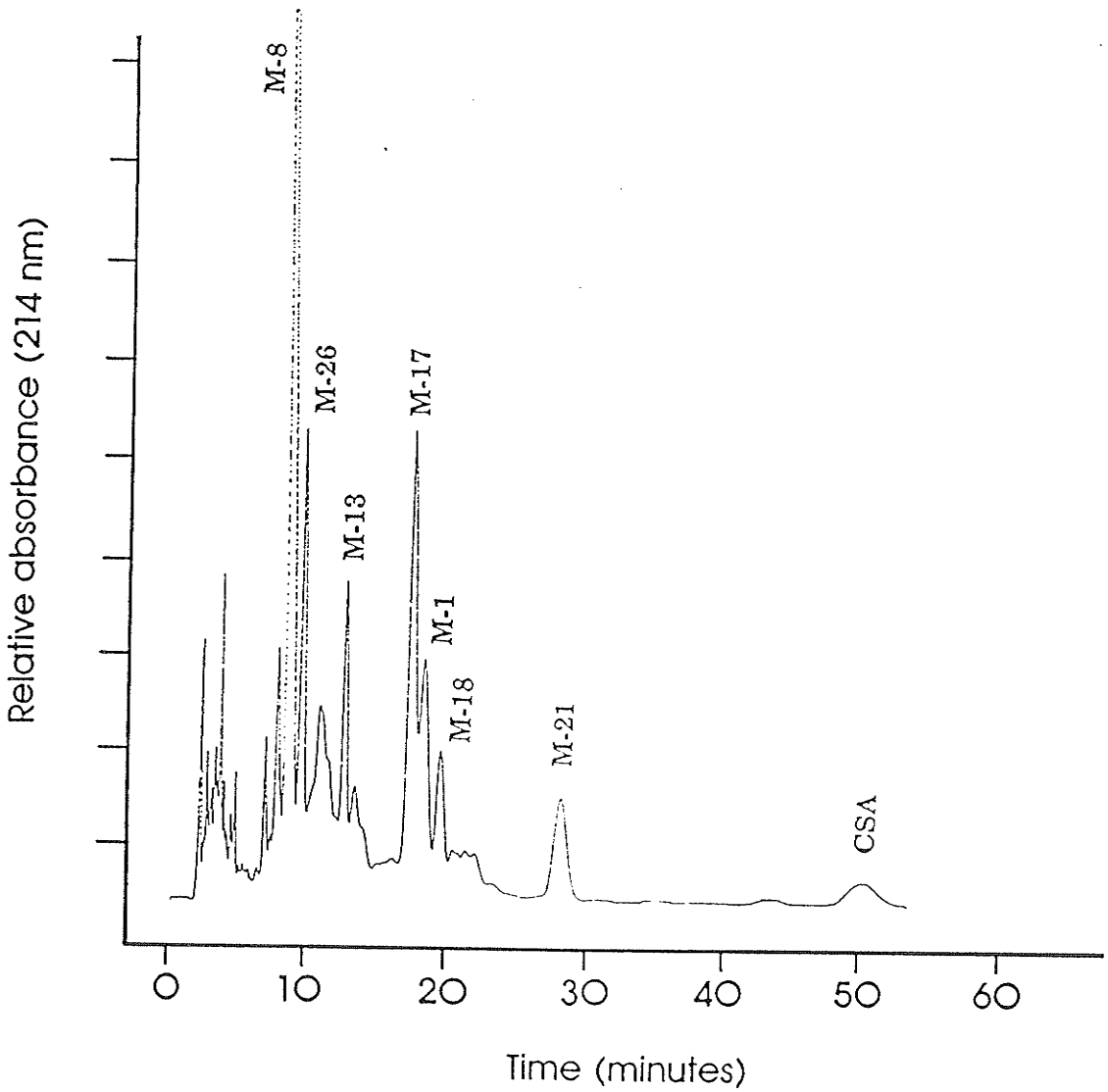

Figure 6. HPLC profile of CsA metabolites isolated from the bile of liver transplant recipients. Bile was obtained by T-tube drainage from liver transplant recipients receiving CsA. The individual metabolites are identified and labelled according to the scheme of Maurer et al (110).

Table 3. Structural characterization of cyclosporine metabolites.

HPLC peak	Metabolite	Molecular weight (Da)	Modification
A	M-8	1234	Hydroxylation AA-1 Hydroxylation AA-9
B	M-26	1234	Hydroxylation AA-1 Hydroxylation AA-9 Cyclization AA-1
C	M-13	1204	Demethylation AA-4 Hydroxylation AA-9
D	M-17	1218	Hydroxylation AA-1
E	Dihydro-M-17	1220	Hydroxylation AA-1 Saturated AA-1
F	M-1	1218	Hydroxylation AA-9
G	M-18	1218	Hydroxylation AA-1 Cyclization AA-1
H	M-21	1188	Demethylation AA-4

compared to the loss of 113 Da for CsA, indicating that an additional oxygen was present in amino acid 1 (AA-1). The proton-NMR spectra confirmed the modification at AA-1, as demonstrated by a shift in the η-CH_3 peak obtained for this amino acid, normally found at 1.62 ppm in CsA. ^{13}C-NMR studies of this metabolite confirmed the above findings. Table 4 summarizes the chemical shifts observed for this as well as other CsA metabolites. Two major alterations were observed occurring at AA-1 and AA-9. The presence of two new peaks at 63.5 and 69.5 ppm correspond to the addition of two hydroxyl groups corresponding to the hydroxylated η-carbon of AA-1 and γ-carbon of AA-9, respectively. There was loss of the η-CH_3 group of AA-1 (17.96 ppm) as well as slight modifications in the chemical shifts of the majority of carbons in AA-1. Similarly, there were alterations in most carbon atoms of AA-9. These findings, along with that observed with FAB/MS and proton-NMR confirmed that this metabolite was a dihydroxylated metabolite of CsA with hydroxylation occurring at AA-1 and AA-9. This metabolite was thus identified, M-8, according to the scheme of Maurer et al (110).

Peak B: The FAB/MS of this metabolite peak indicated a molecular ion species of m/z 1235, corresponding to an increase of 32 Da over CsA, which is consistent with the addition of two oxygen atoms. The presence of a fragment of 1206 Da, which is the result of a loss of 129 Da from the parent ion, indicated that AA-1 was one site of hydroxylation. The proton-NMR spectrum confirmed the modification at AA-1, as evidenced by the loss of the η-CH_3 peak at 1.62 ppm. In

Table 4. ^{13}C-NMR chemical shift data obtained for CsA and its metabolites.

Position			CsA[a]	M-1	M-8	M-17	M-18	M-21	M-26
AA-1	CH_3N		33.97[b]	34.1	33.8	33.7	32.2	33.6	32.2
	H-C	(α)	58.75	58.9	57.6	57.7	57.7	58.5	57.7
	H-C	(β)	74.74	74.5	72.5	72.6	82.0	74.2	82.0
	H-C	(γ)	35.99	36.0	32.6	32.3	36.8	35.6	38.0
	H-C	(δ)	35.63	35.7	32.3	32.0	41.2	35.0	41.3
	CH_3	(γ)	16.76	16.8	17.3	17.5	17.4	16.9	16.8
	H-C	(ε)	129.68	129.5	131.8	132.0	57.0	129.4	57.0
	H-C	(ζ)	126.32	126.2	130.8	131.0	38.1	126.4	38.0
	CH_3	(η)	17.96	17.7	63.5	63.5	60.1	17.8	60.1
AA-2	H-C	(α)	48.86	48.8	48.8	48.8	48.7	49.2	48.8
	H-C	(β)	25.06	25.0	25.1	25.0	25.2	24.9	24.9
	H-C	(γ)	9.93	9.9	9.8	9.9	9.8	9.8	9.8
AA-3	CH_3N		39.40	39.5	39.4	39.3	39.5	40.6	39.4
	H-C	(α)	50.37	50.3	50.1	50.1	50.0	52.0	49.8
AA-4	CH_3N		31.32	31.3	31.3	31.3	31.1	--[c]	31.3
	H-C	(α)	55.51	55.4	54.8	54.8	54.7	55.3	55.0
	H-C	(β)	35.99	36.1	36.0	36.0	35.9	35.5	36.0
	H-C	(γ)	24.90	24.9	24.9	25.0	25.0	24.8	24.7
	CH_3	(δ)	23.49	23.4	23.5	23.7	23.7	23.3	23.4
	CH_3	(δ')	21.18	21.1	21.0	21.3	21.5	21.0	21.5
AA-5	H-C	(α)	55.39	55.3	55.6	55.6	54.3	55.0	55.0
	H-C	(β)	31.17	31.0	31.2	31.3	31.0	31.2	31.2
	CH_3	(γ)	19.81	19.9	19.8	19.9	19.5	19.6	19.8
	CH_3	(δ)	18.48	18.5	18.3	18.3	18.5	18.6	18.3
AA-6	CH_3N		31.53	31.5	31.5	31.5	31.5	31.4	31.3
	H-C	(α)	55.31	55.6	55.4	55.3	54.1	54.9	54.6
	H-C	(β)	37.41	37.5	37.5	37.5	37.5	37.2	37.8
	H-C	(γ)	25.40	25.6	25.3	25.2	25.6	25.2	25.4
	CH_3	(δ)	23.87	23.9	23.9	24.0	23.9	23.8	23.9
	CH_3	(δ')	21.93	22.0	21.7	21.9	21.8	22.0	22.0
AA-7	H-C	(α)	48.69	48.7	48.6	48.3	48.5	48.6	48.8
	H-C	(β)	16.07	16.1	15.8	15.4	15.8	16.1	15.4
AA-8	H-C	(α)	45.20	45.2	44.8	44.9	45.0	45.0	45.0
	H-C	(β)	18.19	18.0	17.6	17.9	17.8	18.1	17.1

Table 4. ^{13}C-NMR chemical shift data obtained for CsA and its metabolites. (continued)

Position			CsA	M-1	M-8	M-17	M-18	M-21	M-26
AA-9	CH_3N		29.65	29.7	29.8	29.7	29.8	29.3	29.9
	H-C	(α)	48.30	47.3	47.2	48.1	48.0	48.2	47.2
	H-C	(β)	39.04	42.6	42.6	39.1	38.5	39.0	42.6
	H-C	(γ)	24.70	69.6	30.5	24.8	24.8	24.7	69.5
	CH_3	(δ)	23.74	30.5	30.5	23.8	23.8	23.7	30.5
	CH_3	(δ')	21.86	30.2	30.2	21.4	21.9	21.8	30.2
AA-10	CH_3N		29.83	29.9	30.0	30.0	29.9	29.7	29.9
	H-C	(α)	57.54	57.5	58.2	58.3	58.1	57.5	58.0
	H-C	(β)	40.73	40.3	40.2	40.6	40.8	40.8	40.2
	H-C	(γ)	24.55	24.5	24.7	24.3	24.5	24.5	24.5
	CH_3	(δ)	23.85	23.8	23.9	23.8	23.8	23.8	23.8
	CH_3	(δ')	23.38	23.0	23.2	23.5	23.3	23.3	23.1
AA-11	CH_3N		29.81	29.9	29.9	29.9	29.9	29.9	29.8
	H-C	(α)	57.93	57.7	58.4	58.5	58.3	58.1	58.7
	H-C	(β)	29.05	29.1	29.0	29.1	29.2	28.9	29.0
	CH_3	(γ)	18.75	18.7	18.8	18.8	19.0	18.8	19.1
	CH_3	(δ)	20.26	20.1	20.2	20.3	20.6	20.0	20.5

[a] Literature values reported by Kessler et al (97).

[b] Value represents the chemical shift (δ) in ppm.

[c] No signal at δ-value of respective carbon atom of CsA.

addition to confirming the hydroxylation at AA-1, ^{13}C-NMR also confirmed hydroxylation of AA-9, as evidenced by the new peaks at 60.1 and 69.5 ppm corresponding to the hydroxylated η-carbon of AA-1 and γ-carbon of AA-9, respectively (Table 4). Severe alterations were observed in the chemical shifts of the carbon atoms of AA-1, including the loss of the alkenyl carbons of AA-1 (129.68 and 126.32 ppm for CsA). These modifications are consistent with cyclization of AA-1. The ^{13}C-NMR spectrum of this metabolite was remarkably similar to that of M-18, which undergoes similar cyclization of AA-1 (see below). This metabolite was thus identified as M-26 according to the scheme of Maurer et al (110).

Peak C: The protonated molecular ion was at m/z 1205 (MH^+) Da, which is a 2 Da increase over CsA. This is due to the addition of one oxygen (+16 Da) and loss of one methyl group (-14 Da). The presence of a fragment with a m/z of 1092 Da (loss of 113 Da) indicated that there was no modification on the side chain of AA-1. This was confirmed by proton-NMR, in which no shift in the η-CH_3 peak of AA-1 was observed. A loss of one of the seven N-CH_3 peaks corresponding to AA-4 was observed. Also 5 N-CH_3 doublets were present, indicating that demethylation had occurred at AA-4. Unfortunately, insufficient amounts of this metabolite were obtained which prevented ^{13}C-NMR analysis. This metabolite was identified as M-13 according to the scheme of Maurer et al (110).

Peak D: The protonated molecular ion was at m/z 1219 (MH^+) Da,

corresponding to a 16 Da increase over CsA and attributed to the addition of one oxygen atom. The presence of a fragment of 1090 Da, which is the result of a loss of 129 Da from the parent ion, indicated that hydroxylation had occurred at AA-1. Proton-NMR demonstrated a loss of the η-CH_3 peak of AA-1. This was confirmed by ^{13}C-NMR (see Table 4), in which there was a loss of this peak (17.96 ppm) and the formation of a new peak at 63.5 ppm which corresponds to the formation of a hydroxyl group as a result of hydroxylation at AA-1. This metabolite was identified as M-17 according to the scheme of Maurer et al (110). The FAB/MS and proton-NMR spectra are shown in Figure 7. A comparison of the ^{13}C-NMR spectra of CsA and M-17 is shown in Figure 8.

Peak E: The protonated molecular ion was at m/z 1221 (MH^+) Da, which indicated an increase of 18 Da over CsA. This was considered to be due to the addition of one oxygen (+16 Da) and the addition of two hydrogen atoms (+2 Da). The presence of a 1090 Da fragment (loss of 131 Da) indicated that the additional oxygen and two hydrogen atoms were contained in AA-1. The proton-NMR confirmed the addition of this oxygen, as evidenced by the loss of the η-CH_3 group. This metabolite was tentatively identified as a hydroxylated CsA metabolite in which the double bond of AA-1 is saturated, and identified as dihydro-M-17, according to similar data reported by Meier et al (135). The lack of sufficient quantities of this metabolite prohibited ^{13}C-NMR analysis. Such studies would have positively confirmed the identification of this metabolite.

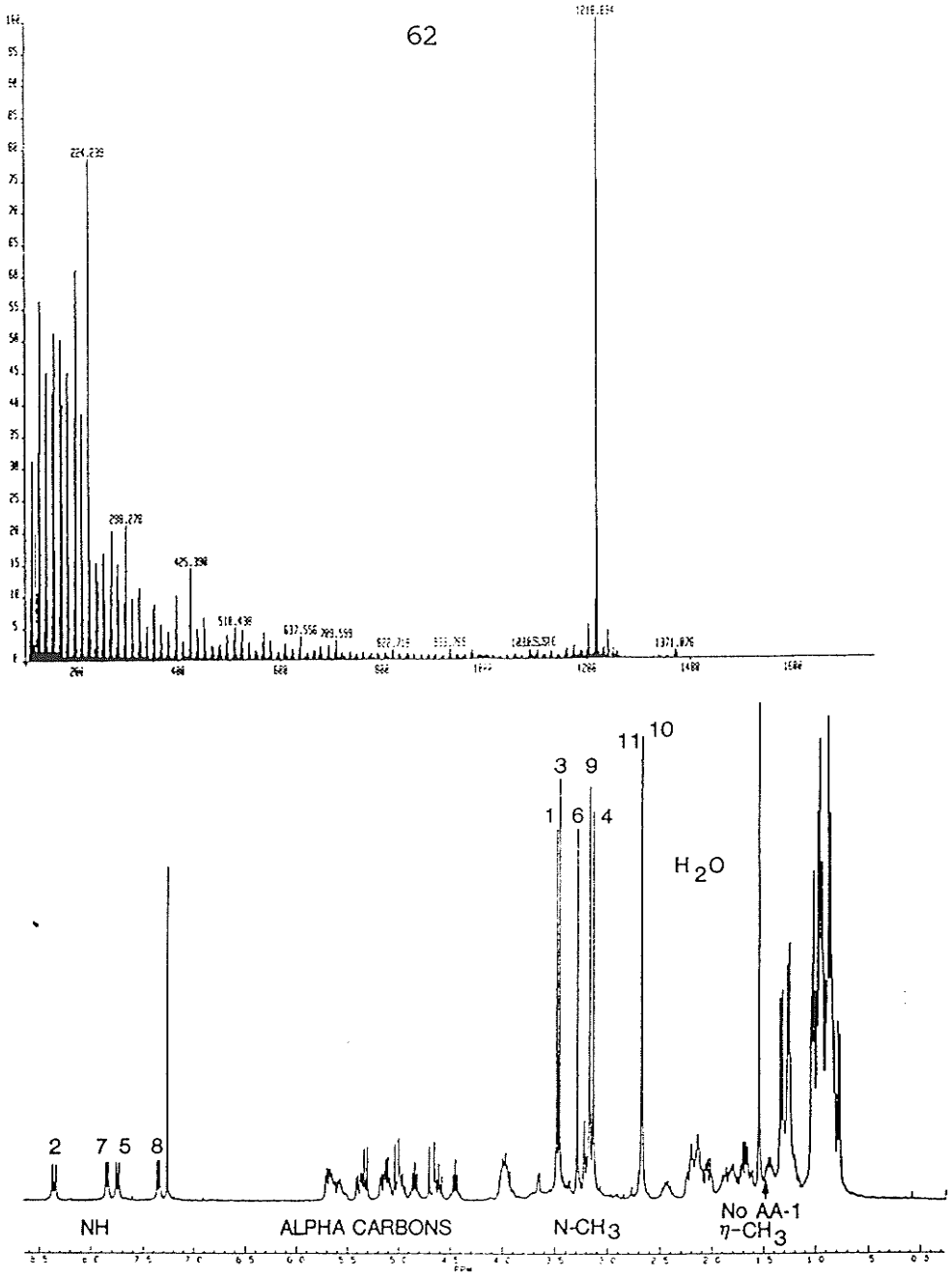

Figure 7. FAB/MS and proton-NMR spectra of cyclosporine metabolite, M-17. The FAB/MS spectrum of M-17 (Top) indicated a parent ion of MH^+ 1218 Da. The proton-NMR spectrum (Bottom) of M-17 is depicted. The identity of the individual protons of the amino acids are labelled. Note that the proton peak usually seen for the η-CH_3 of amino acid 1 (1.62 ppm) is absent, thus indicating a structural modification at this location.

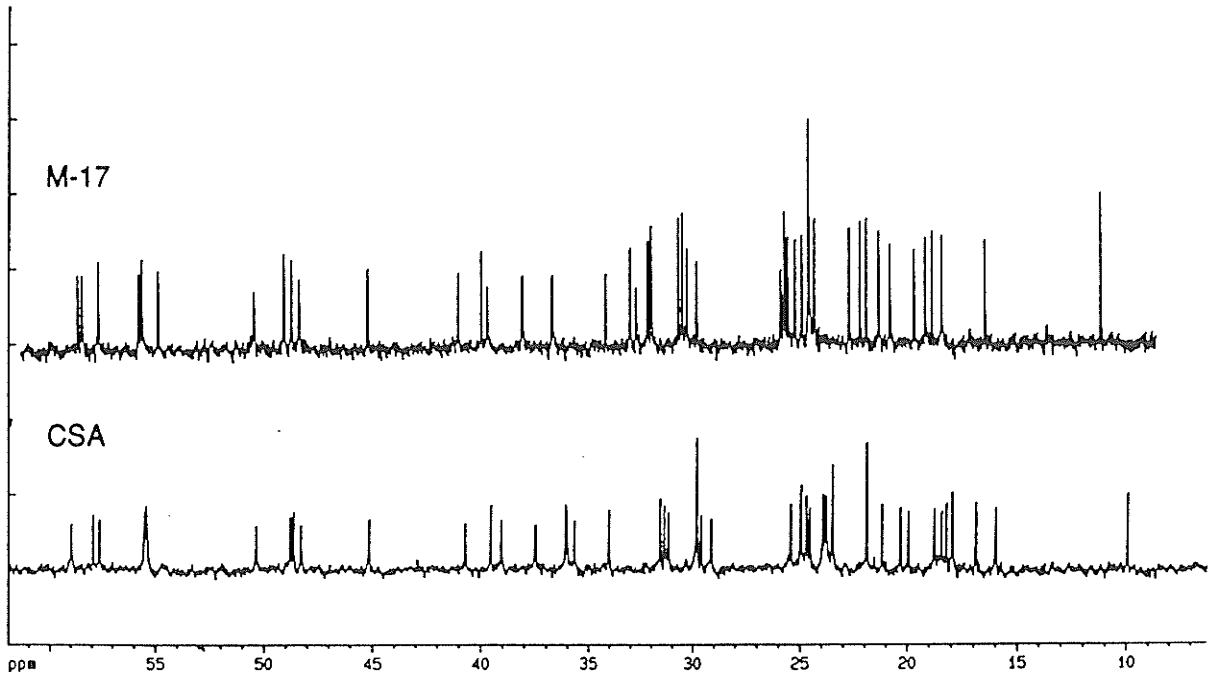

Figure 8. ^{13}C-NMR spectrum of cyclosporine metabolite, M-17. The ^{13}C-NMR spectrum (10 to 60 ppm) of M-17 and parent cyclosporine are compared. Note the similarity of the two spectra. The only differences between the two spectra occur in the shifts of the carbon atoms of amino acid 1, the site where hydroxylation occurs (the value for the chemical shifts are presented in Table 4).

Peak F: The protonated molecular ion was at 1219 Da (MH^+), which is a 16 Da increase over CsA. The presence of a 1106 Da fragment (loss of 113 Da) indicated that no modification had occurred at AA-1. This was confirmed by proton-NMR (no loss of the η-CH_3 peak at 1.62 ppm). ^{13}C-NMR indicated that the only changes observed were at AA-9, thus indicating that hydroxylation had occurred at this site (Table 4). The presence of a peak at 69.5 ppm corresponding to the formation of a hydroxyl group confirmed this finding. This metabolite was identified as M-1 according to the scheme of Maurer et al (110).

Peak G: The protonated molecular ion was at 1219 Da, which was a 16 Da increase over CsA, which was ascribed to the addition of one oxygen atom. The presence of a fragment with m/z 1090 Da (loss of 129 Da) indicated that the oxygen was contained at AA-1. The shift of the η-CH_3 peak confirmed this modification. ^{13}C-NMR analysis revealed significant modification of all carbon atoms of AA-1 (Table 4). The loss of the η-CH_3 peak of AA-1 (17.96) and the presence of a peak at 63.5 ppm confirmed hydroxylation at AA-1. Because of the excessive alterations of the chemical shifts of the carbon atoms of AA-1, cyclization of AA-1 was thought to occur. This metabolite was identified as M-18 according to the scheme of Maurer et al (110).

Peak H: The protonated molecular ion was at m/z 1189, a 14 Da decrease from CsA, which was ascribed to the loss of one methyl group. A fragment of 1076 Da was identified (-113 Da) which indicated that no modification had occurred at AA-1. The proton-NMR indicated the loss

of one of the $N-CH_3$ singlets corresponding to amino acid 4, with an additional NH doublet being observed. ^{13}C-NMR analysis revealed the absence of the CH_3N peak of AA-4, thus confirming the demethylation (Table 4). No other modifications were observed. This metabolite was identified as M-21 according to the scheme of Maurer et al (110). The FAB/MS and proton-NMR spectra of this metabolite are shown in Figure 9.

In addition to the eight major peaks discussed above, there were several other minor peaks observed which were suspected to be CsA metabolites. However, definitive structural characterization could not be performed due to their limited quantities. For this reason no attempt was made to isolate and identify them. Rather, it was decided to use only well-characterized metabolites for subsequent studies (see Chapters 2 through 4).

The purity of the isolated metabolites was assessed using a combination of several techniques. Initially purity was assessed as the metabolite eluting as a single peak by HPLC. Subsequently, purity was assessed using FAB/MS and NMR by observing the spectra for irregularities. For example, with FAB/MS, two metabolites with different molecular weights would appear as two parent ions. To assess the sensitivity of this method in the detection of impurities, 100 µg aliquots of CsA (MH^+ 1203) were spiked with increasing amounts (0 to 10 µg) of M-17 (MH^+ 1219). The samples were analyzed by FAB/MS and the spectra examined. By measuring peak heights of the two parent ions in question, between 2 and 3% impurities of M-17 could be

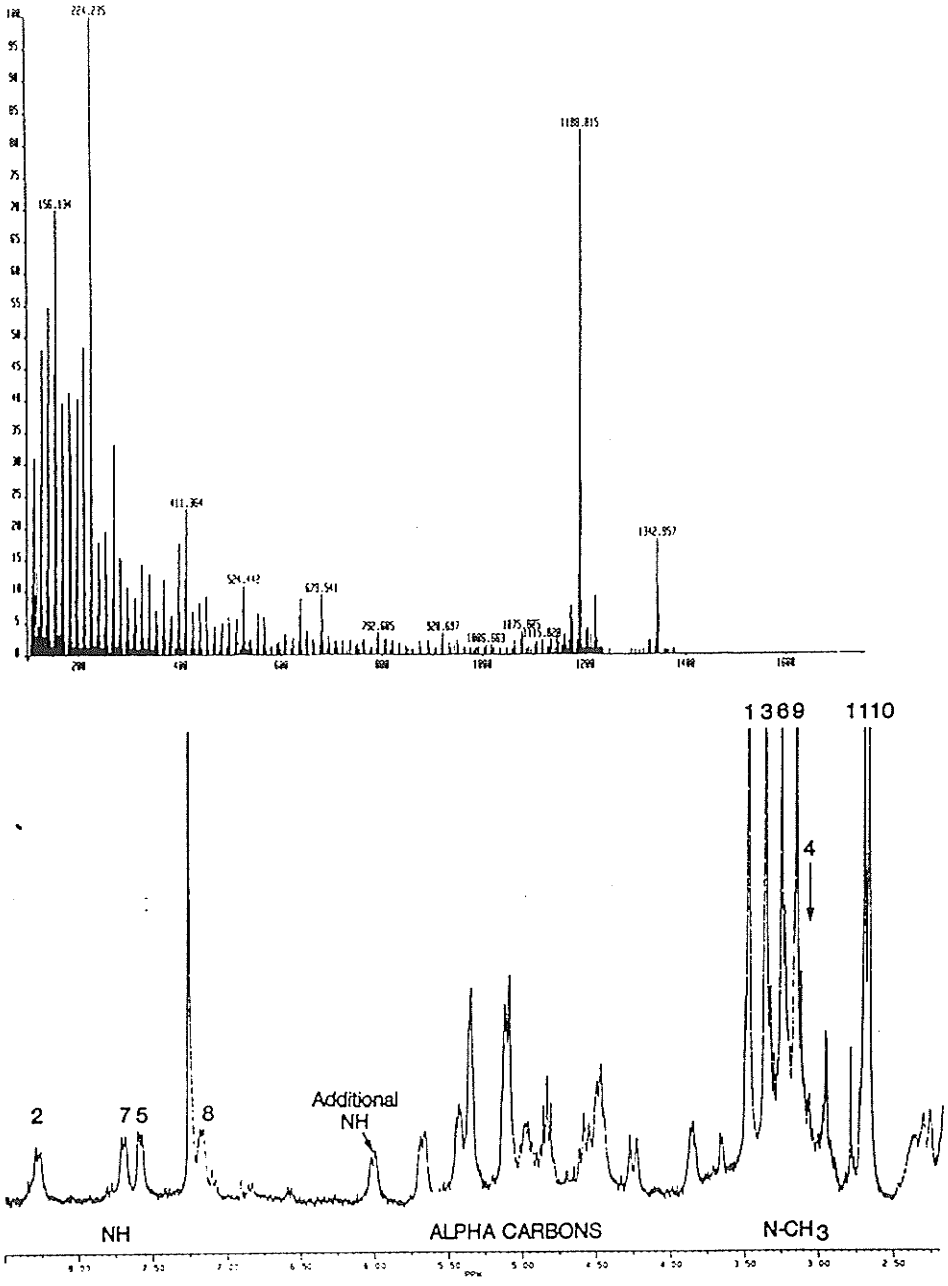

Figure 9. FAB/MS and proton-NMR spectra of cyclosporine metabolite, M-21. The FAB/MS spectrum (Top) indicates a parent ion of MH^+ 1188 Da. The proton-NMR spectrum (Bottom) indicates the loss of the $N-CH_3$ group corresponding to amino acid 4 (arrow) and the presence of the extra N-H group.

Table 5. Cross-reactivity[a] of CsA metabolites with the Sandoz selective and non-selective whole-blood radioimmunoassays for cyclosporine.

Metabolite	Concentration of metabolite added (μg/L)	% cross-reactivity Sandoz selective $\bar{x}$[b]	SD	Sandoz non-selective $\bar{x}$	SD
M-1	5000	8.4	0.49	--	--
	1000	10.1	0.24	72.9	18.3
	500	11.8	0.98	66.5	7.8
	Mean	10.1 (3)[c]		69.7 (34)	
M-8	5000	<1	--	--	--
	1000	<1	--	44.8	3.2
	500	<1	--	56.5	4.2
	Mean	<1 (<1)		50.7 (24)	
M-13	5000	<1	--	4.6	0.73
	1000	<1	--	2.4	0.74
	500	<1	--	1.0	--
	Mean	<1 (<1)		2.7 (3)	
M-17	5000	2.0	0.06	--	--
	1000	1.8	0.12	74.2	5.3
	500	3.9	0.90	92.0	19.8
	Mean	2.6 (<1)		83.1 (76)	
M-18	5000	<1	--	--	--
	1000	<1	--	45.0	6.1
	500	<1	--	54.4	7.6
	Mean	<1 (<1)		49.7 (52)	

Table 5. Cross-reactivity of CsA metabolites with the Sandoz selective and non-selective whole-blood radioimmunoassays for cyclosporine. (continued)

Metabolite	Concentration of metabolite added (µg/L)	% cross-reactivity Sandoz selective $\bar{x}$	SD	Sandoz non-selective $\bar{x}$	SD
M-21	5000	4.2	0.33	9.8	1.0
	1000	4.6	0.16	10.3	1.8
	500	5.1	0.08	10.5	0.9
	Mean	4.6 (3)		10.2 (6)	
M-26	5000	<1	--	--	--
	1000	<1	--	53.2	5.9
	500	<1	--	70.9	4.9
	Mean	<1 (<1)		62.1 (68)	
CsA	500	96	2.9	98	29.6
	250	90	1.6	101	15.4
	100	88	4.5	88	13.0
	Mean	91 (100)		96 (100)	

[a] Cross-reactivity was determined as (metabolite recovered as measured as CsA/metabolite added) x 100.

[b] Results represent a mean of duplicate experiments performed on three separate days.

[c] Values in parentheses indicate the cross-reactivity of the selective or non-selective monoclonal antibody with CsA and its metabolites (Sandimmune RIA Package Insert, Sandoz Inc.).

detected in the CsA. Using the above samples, a similar experiment was carried out using proton-NMR. In this case, the spectrum exhibited additional N-H doublets in the region of 6.5 to 8.5 ppm. In this case >5% impurities could be detected. Thus, using the above procedures, the isolated metabolites were at least 95 to 97% pure.

C. CROSS-REACTIVITY OF CsA METABOLITES

Table 5 summarizes the cross-reactivity data obtained for the isolated metabolites with the Sandoz selective and non-selective monoclonal antibodies. The isolated metabolites exhibited little cross-reactivity with the selective antibody; M-1 exhibited up to 10% cross-reactivity, whereas M-17 and M-21 had values of 3 and 5%, respectively. The remaining metabolites examined had <1% cross-reactivity. The metabolites exhibited cross-reactivity to varying degrees with the polyselective antibody. M-17 was the most cross-reactive exhibiting a value of >80%. Most metabolites examined exhibited cross-reactivities >50%: the exception were M-13 and M-21, which had values <10%. These values are similar to those previously reported by Sandoz (unpublished data) and listed in the table. The only major discrepancy was the cross-reactivity of M-1, which exhibited increased cross-reactivity in this study.

DISCUSSION

A prerequisite for examining the role of CsA metabolites in overall immunosuppression and toxicity requires the procurement of CsA metabolites with known structure and purity. Before meaningful

conclusions about their role can be elucidated, verification of both structure and purity of CsA metabolites is mandatory.

The use of FAB/MS and both proton- and ^{13}C-NMR allowed for the determination of the identity and structure of eight isolated CsA metabolites. These eight metabolites were chosen since they were the most abundant, and sufficient amounts could be isolated to adequately determine their structure as well as to perform subsequent biological studies. Several other minor metabolites were observed but were not isolated since sufficient amounts could not be practically obtained for proper characterization.

The structure and identity of the eight metabolites isolated was determined using the rationale previously discussed. The metabolites identified were M-1, M-8, M-13, M-17, M-18, M-21, M-26, and dihydro-M-17. The modifications observed in these metabolites included hydroxylation, demethylation, saturation of the double bond of AA-1, and cyclization of the side chain of AA-1. Metabolites resulting from hydroxylation of AA-1 included M-17, M-18, M-8, and M-26. Hydroxylation at AA-9 was observed in M-13, M-8, and M-26. Demethylation of AA-4 was demonstrated for M-21 and M-13. Finally, cyclization of the side chain of AA-1 was observed in M-18 and M-26. In addition to the major CsA metabolites previously identified by others, a novel monohydroxylated metabolite in which the double bond in amino acid one was saturated was identified. The structure of this metabolite was consistent to that of dihydro-M-17. This was confirmed by both FAB/MS and proton-NMR. Additionally, saturation of the double bond would produce an additional 2 Da increase in molecular weight.

The identity of this metabolite has been previously reported by Wang et al (129) and Meier et al (135).

FAB/MS has been used by several investigators to examine the structure of CsA metabolites (110,123-126,135,136,191,192). This procedure can give reliable information as to the molecular weight and purity of isolated metabolites. However, its utility in identification of individual metabolites is limited due to the fact that many metabolites possess the same molecular weight. For example, all monohydroxylated metabolites of CsA have a molecular weight of 1234 Da. In addition to the parent ion, a minor ion 113 Da less than that of the parent ion is observed for CsA. This fragment results from the loss of the side chain of amino acid 1. Thus, modification of this side chain will change the size of this fragment. For example, hydroxylation at amino acid 1 (as occurs in M-17) will result in formation of a fragment 129 Da less than the parent ion of M-17 (not 113 Da less, as seen for CsA), confirming modification at this amino acid.

NMR has proven useful for the determination of structure of CsA metabolites, particularly ^{13}C-NMR. Proton-NMR can provide useful information on the site of certain structural modifications. However, in many cases it cannot provide enough information to positively confirm structural modifications of isolated CsA metabolites. This procedure can only detect modification of the η-CH_3 group of amino acid 1 and demethylation of amino acid 4. Hydroxylation at amino acid 1 (as occurs with M-17) will result in loss of the η-CH_3 peak at 1.62 ppm normally seen for CsA. Demethylation at amino acid 4 will result

in the loss of the $N-CH_3$ peak for AA-4 and the subsequent formation of an extra N-H doublet for this site.

^{13}C-NMR analysis provides the most structural information for isolated CsA metabolites. A disadvantage with this method is the large sample requirement. Even with a 300 mHz instrument, greater than 1 mg of sample is required to obtain an adequate spectra. This limits the usefulness of the method to those metabolites which can be isolated in such quantities. The ^{13}C-NMR chemical shifts for all carbon atoms found in CsA can be found in the literature (97). This allows assignment of the chemical shifts for isolated CsA metabolites. Any modification, such as those seen with CsA metabolites, will result in a modification of the ^{13}C-NMR spectra. Thus, the modifications seen in CsA metabolites can be positively identified. For example, demethylation at amino acid 4 (as seen with M-21) will result in loss of the $N-CH_3$ peak for this amino acid, normally observed in CsA. Hydroxylation at amino acid 9 (as seen with M-1) will result in conversion of the γ-CH group of this amino acid to γ-C-OH. This will be observed in the spectra as the loss of the γ-CH peak, normally observed for CsA, and the subsequent formation of a new peak with an increased chemical shift, consistent for a hydroxylated carbon atom.

Cross-reactivity of isolated CsA metabolites with various antibodies has been used with limited success for the identification of CsA metabolites (122,127). There are several limitations with such a method. In order to compare the cross-reactivity of an unknown metabolite to a particular metabolite, pure, well-characterized standards are required. In some cases, values for cross-reactivity of

a particular metabolite with a particular antibody are found in the literature. However, the reliability of such data can be suspect due to the unknown purity of the metabolite used to generate the data. Secondly, the imprecision of this method limits the detection of impurities.

Other more novel methodologies have the potential to be used for the characterization of CsA metabolites. Tandem mass spectrometry has played an important role in structural determination of peptides (196) and has the potential to be useful for the characterization of CsA metabolites. A recent report by Bowers et al (197) used this methodology to identify a novel metabolite of CsA, ^{9}hydroxyl ^{9}desmethyl cyclosporine. The use of other NMR techniques, such as two-dimensional NMR (193,198), have the potential to provide detailed three-dimensional conformational and structural information of CsA metabolites. The lack of sufficient quantities of metabolites has prohibited such studies.

The careful determination of the purity of isolated metabolites has often been overlooked in many of the previous studies (110,122,127-131,134-136,191,192). The assessment of purity in some cases was based on the isolated metabolite eluting as a single symmetrical peak by HPLC. This method obviously has its limitations. It will detect impurities of metabolites that differ in their relative retention times for a particular HPLC system, however co-eluting or closely eluting impurities may not be detected. For example, Bowers et al (197) have recently identified a metabolite of CsA that co-elutes with M-17. The use of FAB/MS is of value in indicating the

purity of a particular metabolite. It was found that impurities as little as 2 to 3% could be detected using this method provided the contaminant had a different molecular weight than that of the metabolite of interest. The use of proton-NMR to assess purity was also examined. By examining the spectra for changes in the N-H doublets (6.5 to 8.0 ppm), the presence of other CsA metabolites can be detected. Using this technique, impurities >5% could be detected.

CsA metabolites have been isolated from bile obtained from a number of animal species, including man, using various HPLC methods (110,122,127-131,134-136,191,192). As stated previously, 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. In all instances, specialized chemical techniques such as mass spectrometry and NMR were not used to verify both the structure and purity of the metabolites isolated.

In 1984, Maurer et al (110) isolated nine ether-extractable metabolites of CsA from the urine of dog and man and from rat bile and feces and purified them by HPLC. Structural assignments were mainly based on spectroscopic data (MS, proton- and ^{13}C-NMR). In contrast, studies by Rosano (131,144,145) only used HPLC as a criteria for identification. Lensmeyer (130) reported the isolation and purity of nine CsA metabolites obtained from whole blood. In addition to HPLC, cross-reactivity with polyclonal antibodies were used to assess their identity. No chemical characterization was used to identify the metabolites. Subsequent studies by Hartman (127), Cheung (123), and Wallemacq (128) have used HPLC as well as mass spectroscopic methods

to characterize isolated CsA metabolites. The problem with these studies is that only the molecular weight of the metabolite in question is discerned using mass spectrometry. These studies gave little insight into the structure of CsA metabolites. Furthermore, in most instances the site of hydroxylation, using mass spectrometry alone, cannot be ascertained.

More recent studies, where more detail to structural characterization has been observed, have provided more information as to the identification and purity of isolated CsA metabolites (123,129,134-137,192,197). The structural identity of the metabolites in our study agrees with a recent report by Wang et al (129), who studied metabolites isolated from bile of transplant patients using FAB/MS and proton-NMR. However, no study to date, other than the initial one by Maurer et al (110), has employed ^{13}C-NMR to identify CsA metabolites. As discussed earlier, this technique can provide the most information about the site of chemical modification. It can also give some idea of conformational changes. However, the quantity of material required, as well as the difficulty in obtaining such quantities, has limited such studies.

There have been several novel CsA metabolites which have been identified. There is evidence of a carboxylic acid metabolite (M-203-218). This metabolite arises due to modification of the terminal methyl group of amino acid 1 to a carboxylic acid (125,126). This acidic metabolite of CsA results from modification of the η-CH_3 group of amino acid 1. This group is oxidized to an α,β-unsaturated carboxylic acid. This metabolite has the potential for subsequent

chemical reactions due to the high reactivity of such groups. Further, a sulphate conjugate of CsA has been isolated from human bile and plasma (136,137). This is the first evidence of a conjugated metabolite of CsA. A novel, previously identified, metabolite (M-E) has been identified in the blood of renal transplant patients (134) and shown to possess significant immunosuppressive activity in vitro.

From a clinical standpoint it is important to know whether CsA metabolites possess immunosuppressive and/or toxic properties. CsA is currently administered so that a given dose results in a certain clinical outcome. It is recognized that the clinical response observed for a given dose (degree of immunosuppression or toxicity) does not correlate well with the administered dose. It is thus important that concentrations of CsA in blood be monitored and the results obtained used in conjunction with other laboratory and clinical data to guide in dosage adjustments. The finding that metabolites are immunosuppressive and/or toxic and thus contribute to the overall immunosuppressive state would have important consequences. For a given dose of CsA, besides parent drug producing biological effects, the contributions by metabolites would also have to be considered.

The incorporation of the knowledge of CsA metabolite biological activity would help to guide a more effective dosing of CsA in continuing efforts to minimize the risks of side effects while maximizing immunosuppression. A first step in gaining this knowledge is to obtain well-characterized, purified metabolites. This is essential in attempting to attribute a particular biological function

to a specific metabolite. The studies presented above (also 191,192) have emphasized the importance of obtaining metabolites with known structure and purity. While several investigators have previously characterized CsA metabolites, few have used the protocols presented in this thesis to ensure the identity and purity of isolated metabolites. The availability of such compounds will allow for subsequent investigation into the biological significance of these major CsA metabolites.

IV. CHAPTER 2

IMMUNOSUPPRESSIVE ACTIVITY OF CsA METABOLITES

METHODS

A. PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (199) as previously described (191,192). All procedures were performed using sterile techniques in a laminar flow hood and all solutions used were at room temperature. Heparinized whole blood was obtained via venipuncture from HLA incompatible healthy donors. The blood was diluted 1:1 with sterile saline, layered over Histopaque-1077 (3 parts blood:1 part histopaque) (Sigma Chemical Co, St. Louis, MO, USA), and centrifuged for 30 minutes at 800 xg. The lymphocyte layer was removed and washed 3 times with sterile saline. The isolated lymphocytes were resuspended in 5 mL of media (see below) and cell viability determined by trypan blue dye exclusion. Viable cell numbers were determined using a hemacytometer. A pool of cells from 10 healthy donors was isolated as above, pooled and frozen. This pool was used as a stimulator population for the subsequent primary MLC experiments (see below).

B. PHYTOHEMAGGLUTININ MITOGEN STIMULATION ASSAY

PBMC (1×10^6/mL) were cultured in triplicate in 96 well microtitre plates in RPMI-1640 media (Gibco Laboratories, Grand

Island, NY, USA) containing 10% (v/v) AB (blood group) human serum, 100 U/mL penicillin, and 0.1 μg/mL streptomycin. Twenty μL of CsA, control, or metabolite stocks were added to the appropriate wells and the cells incubated at 37°C in the presence of 5% CO_2. After 48 hours, the cultures were pulsed with 0.3 μCi [^{3}H]-thymidine (Amersham Inc., Oakville, ON) per well. Eighteen hours later the cells were harvested using a cell harvester and the radioactivity determined by liquid scintillation counting.

C. PRIMARY MIXED LYMPHOCYTE CULTURE SYSTEM (1° MLC)

Responder PBMC will proliferate when cultured with PBMC of another HLA phenotype (stimulator). One hundred μL of responder PBMC (1 x 10^6/mL) were cultured with 100 μL of a pool (10 donors) of irradiated (2500 rad) stimulator cells in the media described above. The latter cells were irradiated to prevent their proliferation, but they still had the ability to stimulate the responder cells. Assays were performed in quadruplicate in 96 well microtitre plates. The cells were incubated with 20 μL of CsA, metabolite, or vehicle stocks for six days with an 18 hour terminal pulse of [^{3}H]-thymidine (0.3 μCi/well). The incorporation of [^{3}H]-thymidine was determined as described above.

D. SECONDARY MIXED LYMPHOCYTE CULTURE SYSTEM (2° MLC)

Ten x 10^6/mL responder PBMC were cultured with 10 x 10^6/mL irradiated (2500 rad) stimulator PBMC (pool of 10 donors) in a total volume of 10 mL of complete media for six days. The primed cells were

then washed and recultured in triplicate (5×10^4/100 µL) with the same pool of irradiated stimulator cells (1×10^5/100 µL), with or without CsA or metabolite in 96 well microtitre plates. The cells were incubated for a further 72 hours with a 4 to 6 hour terminal pulse of [^{3}H]-thymidine as described above.

E. PREPARATION OF CsA AND METABOLITE STOCK SOLUTIONS

The CsA and metabolite stock solutions were prepared as follows. The CsA or metabolites were dissolved in 10 µL of 95% (v/v) ethanol containing 20% (v/v) Tween 80 and diluted with RPMI-1640 media to the desired concentration of drug (final ethanol concentration was 0.5%). For a control, RPMI-1640 medium was prepared containing 0.5% (v/v) of the above ethanol/Tween 80 solution. The same stocks were used for all experiments. This concentration of ethanol/Tween 80 was found to have no immunosuppressive effect on any of the above systems. The concentration of CsA and metabolites ranged from 2.0 to 20,000 µg/L. The concentration of the prepared stocks was confirmed by HPLC as previously described. The same stock solutions were used for all experiments.

F. CALCULATION OF RESULTS

The 50% inhibitory concentration (IC_{50}) for CsA and each metabolite was determined by graphical analysis. The final results were expressed as a potency ratio of each metabolite with CsA, i.e. IC_{50} of CsA/IC_{50} of metabolite. All experiments were done on three separate days, and were expressed as a mean and standard deviation.

Statistical analysis was performed using a two-way analysis of variance program.

G. DETERMINATION OF THE IMMUNOSUPPRESSIVE ACTIVITY OF METABOLITE FRACTIONS COLLECTED THROUGHOUT AN HPLC PROFILE

A pool of urine was obtained from renal transplant recipients receiving CsA (10 L). CsA and metabolites were extracted from the urine and chromatographed as previously described. However, instead of manually collecting individual peaks, one minute fractions were automatically collected for the entire chromatographic profile (55 minutes) using a fraction collector. With the chromatographic conditions used, this allowed for collection of all material of interest (the chromatographic profile is seen at the bottom of Figure 10). After several injections (approximately 40), the amount of material in each fraction was calculated as follows: the amount of metabolite of each fraction was determined by comparing the area under the curve (AUC) of the fraction in question to that of internal standard (CsC). In some cases more than one peak was found in each fraction. In this case, the total AUC was used to calculate the amount of material. Each fraction was then analyzed for immunosuppressive activity using a primary MLC using the previously mentioned methods.

RESULTS

Initially, the immunosuppressive activity of a series of fractions obtained over an entire chromatographic profile was

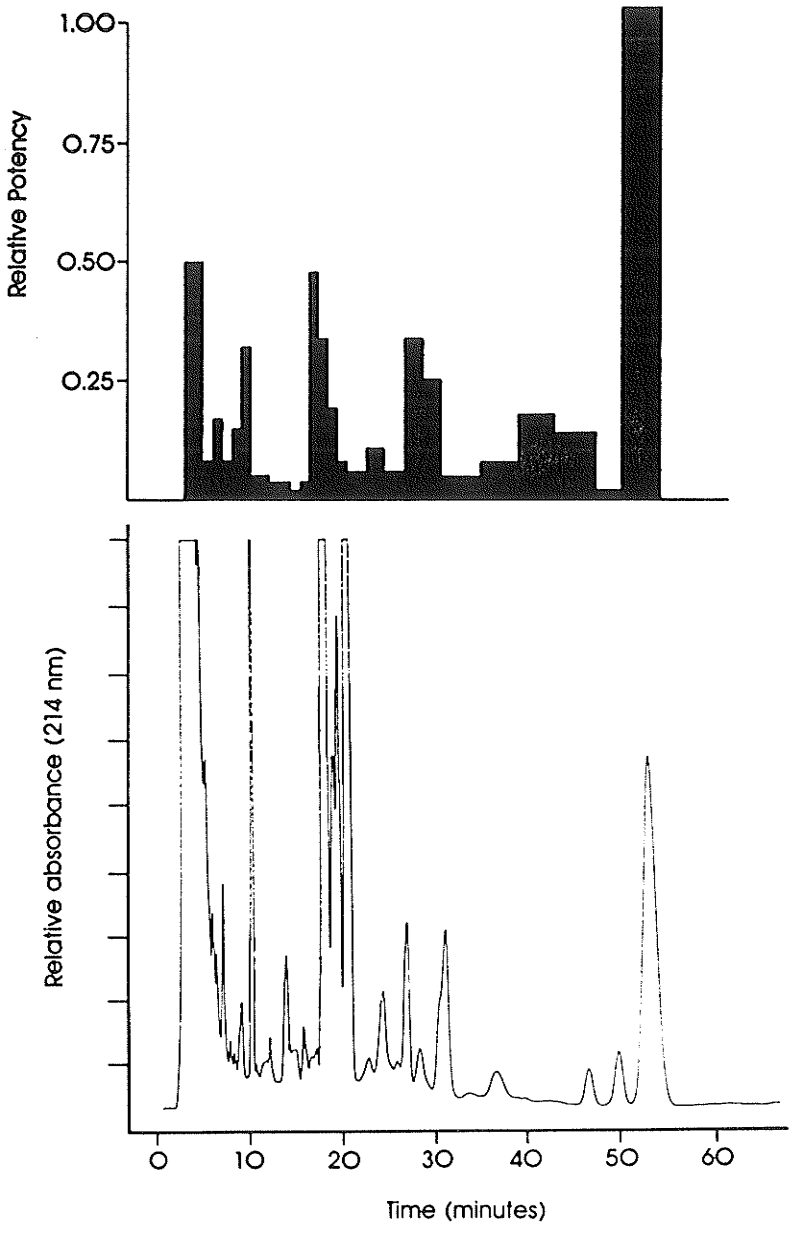

Figure 10. Immunosuppressive activities of various fractions obtained from the entire chromatographic profile of urine. Urine was obtained from several renal transplant recipients and pooled. The results are expressed as a potency ratio compared to an equivalent CsA concentration.

determined. This was undertaken to study the activity of all potential metabolites of CsA, since the possibility exists that certain minor metabolites may exhibit potent immunosuppressive activities. Figure 10 shows the chromatographic profile of the extracted urine (bottom) with the immunosuppressive activity of each corresponding fraction listed above. The immunosuppressive activity was expressed as a potency ratio relative to CsA. No fraction isolated was as active as CsA in inhibiting proliferation in the primary MLC. Interestingly, significant activity was observed in the solvent front and in the area of the peaks corresponding to M-17 and M-1.

Subsequent studies investigated the immunosuppressive activity of the eight major CsA metabolites previously isolated and characterized (see Chapter 1). The immunosuppressive activity was examined using three different assay systems: a primary and secondary mixed lymphocyte culture as well as a phytohemagglutinin-stimulated culture system. All experiments were done in quadruplicate on three different occasions. The results of these studies are summarized below (192). Figure 11 shows a typical dose response curve for CsA and its metabolites in the inhibition of a primary MLR. Note that much lower concentrations of CsA were required to inhibit the response than that required for metabolites. The metabolites are labelled (A-H) according to the chromatogram shown in Figure 5. In addition, the identity of each metabolite peak is listed. Table 6 shows the concentrations of metabolites that result in 50% inhibition of [^{3}H]-thymidine uptake (IC_{50}) in all three test systems. The potency

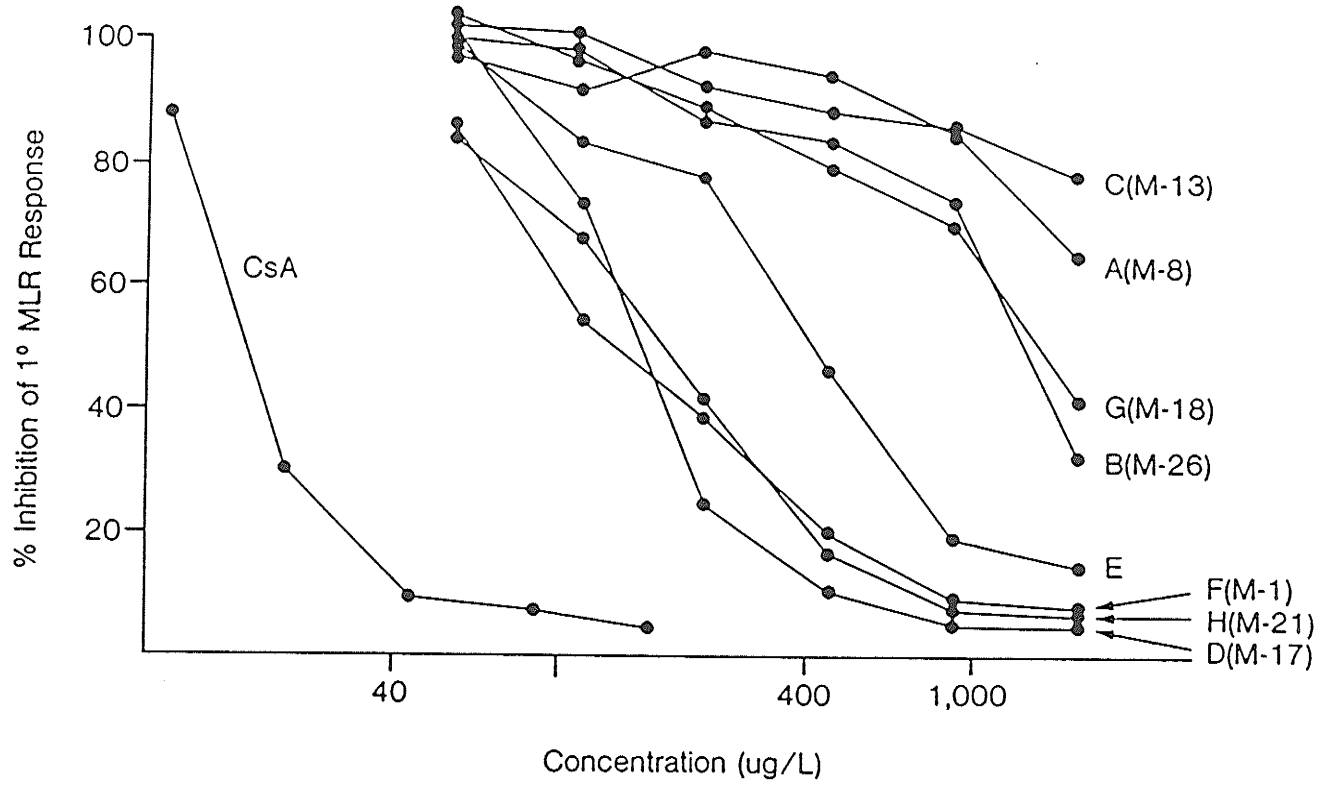

Figure 11. Immunosuppressive activities of CsA and CsA metabolites in a primary mixed lymphocyte culture. The results are expressed as a percent inhibition of the primary MLC response for the various CsA or metabolite concentrations.

Table 6. Immunosuppressive activity of CsA metabolites compared.

Metabolite peak	Metabolite	IC_{50}, µg/L[a] 1° MLC		2° MLC		Phytohemagglutinin	
A	M-8	>2000	(<0.007)[b]	8933 ± 1508	(0.001)	>20,000	(<0.026)
B	M-26	>2000	(<0.007)	7270 ± 870	(0.002)	>20,000	(<0.026)
C	M-13	1667 ± 189	(0.009)	7250 ± 2478	(0.002)	>20,000	(<0.026)
D	M-17	170 ± 7	(0.089)	83 ± 17	(0.16)	20,700 ± 3600	(0.025)
E	Dihydro-M-17	353 ± 81	(0.042)	527 ± 107	(0.02)	11,200 ± 4200	(0.046)
F	M-1	157 ± 19	(0.095)	90 ± 24	(0.14)	5630 ± 120	(0.092)
G	M-18	1500 ± 400	(0.01)	520 ± 106	(0.025)	11,900 ± 2400	(0.044)
H	M-21	183 ± 12	(0.082)	367 ± 26	(0.035)	7300 ± 2400	(0.071)
	CsA	15 ± 3	(1.0)	13 ± 2	(1.0)	520 ± 100	(1.0)

[a] Results are expressed as mean (and SD) of three measurements done on separate days.

[b] Potency of metabolite relative to CsA (CsA = 1.0) is listed in parentheses.

ratios, as defined as the ratio of the IC_{50} of metabolites to that of CsA, are also shown. As can be seen, the IC_{50} of CsA as well as individual metabolites varies among the three systems. Significantly more CsA and metabolite were required to inhibit the phytohemagglutinin mitogen-stimulated system as compared to the alloantigen-stimulated systems. The ranking of immunosuppressive activity of the metabolites relative to CsA also varies among the test systems, with the largest amounts of CsA or metabolites being required to inhibit the PHA system. All metabolites tested had immunosuppressive activities <10% of CsA, except for M-17 and M-1 which exhibited immunosuppressive activities of 16 and 14%, respectively, in a secondary MLC system. In contrast, M-1, M-17, and M-21 exhibited similar potencies in the primary MLC system, whereas M-1 was the most immunosuppressive in the mitogen-stimulated system. From these studies, the overall immunosuppressive potency of the metabolites examined was M-17 > M-1 > M-21 > dihydro-M-17 > M-13 > M-18 > M-8 > M-26.

The immunosuppressive activity of CsA and M-17 alone and in combination was also studied using a primary MLC in order to examine whether a synergistic relationship exists between the two. Two concentrations of M-17 were chosen for the study: one that exhibited negligible immunosuppression (28 μg/L) and one that exhibited 50% immunosuppression (56 μg/L). The concentration of CsA varied between 7 μg/L and 28 μg/L. From the data shown in Figure 12, it can be seen that the overall immunosuppression was additive; the total immunosuppression observed was close to what would be predicted for

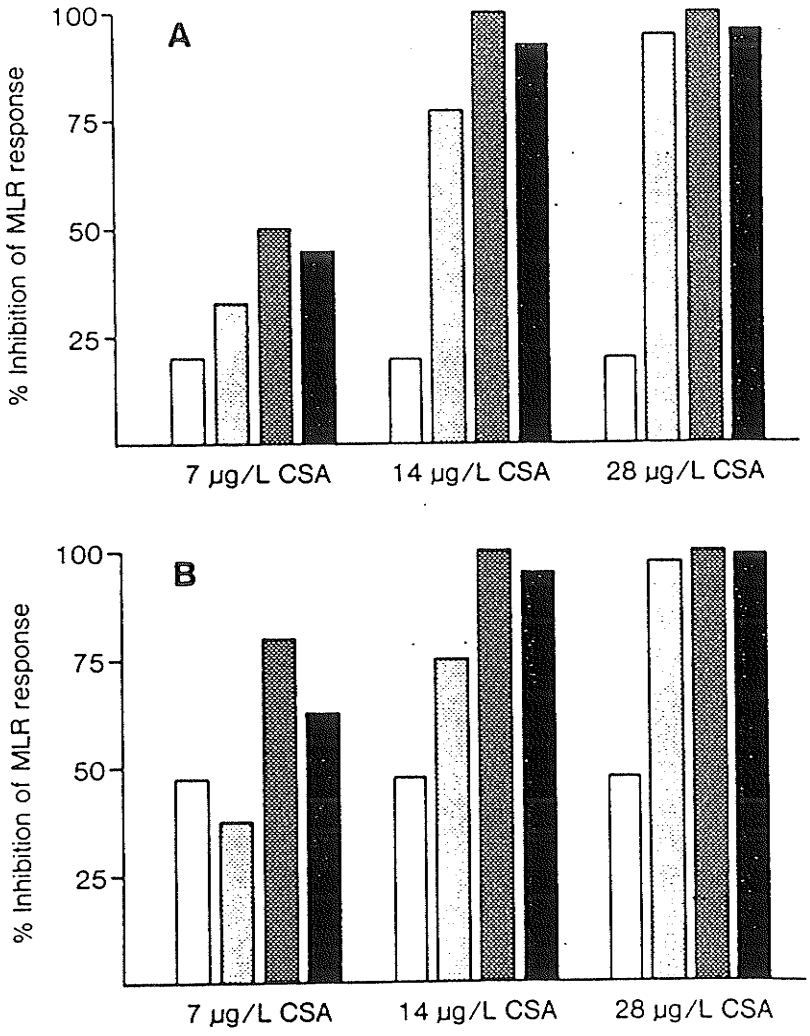

Figure 12. Effect of cyclosporine and M-17 alone or in combination on the inhibition of the MLR response. A. 28 µg/L M-17 (□), CsA (▒), expected combined inhibition (▓), and observed combined inhibition (■). B. 56 µg/L M-17 (□), CsA (▒), expected combined inhibition (▓), and observed combined inhibition (■).

the sum of the two compounds. No synergism was found to exist between the M-17 and CsA.

DISCUSSION

This study was undertaken to assess the in vitro immunosuppressive effects of several well-characterized CsA metabolites (191,192,200). Earlier studies investigating the in vitro immunosuppressive activity of CsA metabolites were poorly standardized (144-150). In most studies no mention was made as to the purity of the metabolites used. In addition, the immunosuppressive activity was expressed several different ways. Often, results were only expressed as [^{3}H]-thymidine incorporation or total cpm, and were not standardized between experiments. Further, IC_{50} values and potency ratios were not determined, thus direct comparison of the activity of a particular metabolite to that of CsA was not possible. The above reasons may explain the great discrepancy in the literature with regards to the immunosuppressive properties of CsA metabolites.

Initially, the immunosuppressive activities of a series of fractions obtained throughout an entire chromatographic profile was determined (Figure 10). Besides the eight metabolites which were chosen to be identified and characterized previously, several other minor metabolites were observed in the chromatogram. These eight major metabolites were found to possess only about 50% of the total immunosuppressive activity determined. A major amount of immunosuppression was attributable to parent CsA. However, other minor metabolites did appear to possess immunosuppressive activity.

Particularly high activity was observed for fractions eluting with or just after the solvent front. The patients from which the urine was obtained were also receiving methyl-prednisolone and azathioprine as part of their immunosuppressive therapy. This high activity may be the result of immunosuppression by these agents or their metabolites which were extracted from the urine. However, the potential for minor metabolites with potent activity eluting in this area cannot be ruled out.

A prerequisite for the study of CsA metabolites is the procurement of CsA metabolites with known structure and purity. In this study the immunosuppressive activities of well-characterized CsA metabolites (see Chapter 1) were examined simultaneously in three separate assay systems. To further minimize error, the same metabolite stocks were used for all experiments. Additionally, the metabolite concentrations of the stock solutions were verified by HPLC. The immunosuppressive activities of the metabolites examined in these studies varied among the assay systems, with most of the metabolites having immunosuppressive activity <10% of that of CsA. In general, the primary metabolites of CsA (M-1, M-17, and M-21) were about 10% as active as the parent drug.

The results presented in this thesis are consistent with recent reports in the literature. Ryffel et al (150) demonstrated that CsA metabolites were 4 to 10 fold less active than CsA in a primary MLC. M-17, the most active metabolite, was 10% as active as CsA. Wallemacq et al (128) also found that M-17 was the most active metabolite, again demonstrating 10% of the activity of CsA in a primary MLC. In earlier

studies, Freed et al (145) found M-17 to possess activity comparable to CsA in a mixed lymphocyte reaction and concanavalin A-stimulated system, followed by M-1 and M-21, which possessed less activity. Similar results were found by Zeevi et al (147-148), who used secondary proliferative responses of cloned alloreactive T cells and transplant biopsy grown lymphocytes. In contrast, Schlitt et al (149) found that all metabolites they examined were not nearly as effective as CsA in inhibiting a primary MLC. Similarly, Hartman and Jardine (127), using a PHA and Con A stimulated system, found that no metabolite was $\geq$10% as effective as CsA in inhibiting the response.

One of the most important mechanisms of action of CsA is the inhibition of IL-2 production. Heidecke et al (201) examined the effect of CsA, M-17, and M-21 on release of this analyte using a bioassay based on the inhibition of IL-2 production. They demonstrated that CsA metabolites had significant effects on the inhibition of IL-2 release. At 1,000 μg/L, CsA inhibited IL-2 production by 97%, whereas M-17 was 80% as effective in inhibiting IL-2 release. However, at more physiological levels (100 μg/L), M-17 resulted in only 15% inhibition, whereas CsA inhibited IL-2 release by 54%. M-21 exhibited less effect on inhibition of IL-2 production (10% at 100 μg/L). Although not as effective as CsA, M-17 and M-21 to a lesser extent did result in significant inhibition of release of this analyte.

The studies reported in this thesis, in which standardized techniques for metabolite isolation, identification, and determination of immunosuppressive activity were used, provided insight into the

immunosuppressive properties of CsA metabolites. These data along with that presented by others in recent studies (121,128,129,191,192), in which more attention is paid to metabolite purity methodological procedures, as well as the expression of results, have allowed for valid conclusions to be made with regards to the in vitro immunosuppressive effects of CsA metabolites. First, metabolites with single modifications [M-17, M-1 (hydroxylation), or M-21 (demethylation)] possess the highest immunosuppressive activity of any CsA metabolite examined, usually 10 to 15% of that for the parent drug. Secondly, metabolites with two or more modifications possess substantially less activity, usually less than 2 to 5% of that for CsA. Thirdly, as a general rule, as metabolite polarity increases, there is a decrease in immunosuppressive activity.

The in vivo data examining the potential immunosuppressive properties of CsA metabolites are limited. Ryffel et al (158) studied the effect of CsA and M-17 in suppressing the production of antibodies against sheep erythrocytes (hemagglutination) in the rat. The IC_{50} of CsA (concentration of drug required for 50% inhibition of antibody formation) was 3 mg/kg/day. In contrast, M-17 had an IC_{50} of >50 mg/kg/day. In subsequent studies, CsA, M-17, M-18, M-21, and M-203-218 was administered to rats subcutaneously at 50 mg/kg for 10 days (157). CsA resulted in significant reduction of thymus weight and severely hampered the production of antibodies to sheep erythrocytes as evidenced by a decreased hemagglutination titre as compared to control animals. In contrast, there was no difference between the control animals and those receiving any of the

metabolites, thus suggesting that CsA metabolites have little in vivo immunosuppressive activity.

The effectiveness of CsA therapy may depend on the immunosuppressive effects of combinations of individual metabolites and the parent compound. It is possible that low levels of CsA in combination with CsA metabolites may result in adequate immunosuppression, whereas concentrations of CsA or any of its metabolites at such levels would not result in adequate immunosuppression. Such a synergism between CsA and its metabolites has been implied. Zeevi et al (148) have suggested a synergistic effect between CsA and its major metabolite, M-17. In our studies no synergistic effect between CsA and M-17 was observed. The reason for this discrepancy is not known, but it may reside in the way in which their results were calculated. A synergistic effect between CsA and a new immunosuppressant agent, FK506, has been reported (202). However, little is known about the mechanism of synergism between the agents, but it may be related to the binding of these drugs to lymphocyte membranes.

The overall contribution of CsA metabolites to immunosuppressive activity in vivo still remains to be determined. It is difficult to extrapolate activity measured with single concentrations of metabolites in vitro to the situation in patients, where many metabolites in different time-dependent concentrations may synergistically act with CsA on their target. In vivo studies in which purified metabolites alone or in combination are administered, with allograft rejection being monitored, are required before

significant conclusions can be drawn regarding the role of CsA metabolites in overall immunosuppression. These studies have not been undertaken due to the difficulty in obtaining significant quantities of metabolites.

The results of this thesis suggest that CsA metabolites are less immunosuppressive than the parent drug and thus may not significantly contribute to overall immunosuppression in patients receiving the drug. Most likely they contribute to the overall immunosuppressive picture in a dose-dependent fashion, however to a much lesser extent than CsA. In transplant recipients with a relatively stable metabolite/parent drug ratio, specific measurement of metabolites is not justified for guiding the dosage of CsA. However, in certain instances such as in liver transplantation, metabolite levels can be extremely high and variable. Often, metabolite levels exceed those of the parent drug greater than 10 fold. In these situations, CsA metabolites may have a significant contribution to overall immunosuppression. The role of CsA metabolites in CsA toxicity, particularly nephrotoxicity, is not known. Finding of a metabolite that possesses some active immunosuppressive properties but which exhibits much less toxic properties than that of CsA would be of clinical relevance. Thus, further studies are required to assess the toxic properties of CsA metabolites.

V. CHAPTER 3

PHARMACOKINETICS AND BIOTRANSFORMATION OF THE CYCLOSPORINE METABOLITE M-17 IN THE RABBIT

METHODS

A. PHARMACOKINETIC STUDIES

The single dose pharmacokinetics of CsA and M-17 were determined (203). CsA (Sandimmune, Sandoz Inc., Basel, Switzerland) was administered via a single i.v. injection to three New Zealand White rabbits (2.5 to 3.5 kg) at a dose of 1.0 mg/kg. CsA was diluted with sterile saline and administered (total volume 2 mL) over 3 minutes into the right marginal ear vein. The rabbits were housed in individual cages with food being restricted during pharmacokinetic analysis. Immediately before CsA infusion, an in-line catheter and heparin lock were placed into the left marginal ear vein to facilitate blood sampling.

Heparinized whole blood (500 to 600 μL) was obtained prior to infusion (time zero) and at the following times after administration of CsA: 3,6,10,20,30,45,60,90 minutes, and 2,3,4,6,8,10,12,18,21,and 24 hours. All samples were stored at 4°C until CsA concentrations were determined.

M-17 was administered to three additional rabbits using the same protocol described for CsA infusion with the following modifications: M-17 was solubilized in 2 mL of 50% (v/v) ethanol/sterile water, and blood samples were obtained as above up to 12 hours.

B. MEASUREMENT OF CsA AND METABOLITES

CsA and M-17 concentrations were measured by HPLC, as previously described, with CsC used as the internal standard.

C. CALCULATION OF RESULTS

Drug concentration vs time was plotted on semi-logarithm graph paper. Model-independent parameters were calculated with the respective blood concentration time data. These included total body clearance (Cl) and steady state volume of distribution (VDss). The terminal disposition constant ($T_{\frac{1}{2}}$ terminal) was obtained from the slope of the terminal portion of the blood concentration time curve. Model-independent parameters were calculated by Dr. N. Honcharik (Department of Pharmacy, Health Sciences Centre) using the computer program PKCALC (204).

D. PHARMACOKINETIC TERMINOLOGY (205)

1. Elimination half-life ($T_{\frac{1}{2}}$)

The half-life of a drug after distribution equilibrium has been obtained. Each half-life is the time interval that results in elimination of 50% of the drug present in the body.

$$T_{\frac{1}{2}} = \frac{0.693}{\beta} \qquad \beta = \text{terminal disposition constant}$$

2. Volume of distribution (Vd)

Volume of distribution of a drug provides an estimate of the extent of its distribution through body-fluid compartments and its uptake by tissues. A large volume of distribution indicates wide distribution and/or extensive tissue uptake.

$$Vd = \frac{Dose}{\beta \cdot AUC}$$ AUC = area under the blood concentration curve

3. Clearance (Cl)

Clearance is an index of drug elimination from the central compartment. This clearance is due to hepatic biotransformation and excretion by the kidney or in the feces.

$$Cl = \frac{Dose}{AUC}$$

E. DETERMINATION OF METABOLITES IN URINE AND BILE

Twelve hours after administration of M-17, the rabbits were sacrificed. Heparinized whole blood was obtained by cardiac puncture, urine was obtained from the bladder, and bile from the gall bladder. The fluids were extracted and assayed for M-17 as well as for the presence of other CsA metabolites by HPLC using previously described procedures. Previously isolated and characterized metabolites were used as standards for the identification of metabolites present in the various fluids. The relative retention times for the metabolite standards with respect to CsC were determined and compared to that obtained for the peaks observed in the chromatograms.

RESULTS

The pharmacokinetic profile for CsA and M-17 for the rabbits is shown in Figure 13. In each case three rabbits were used for either CsA or M-17. The detailed calculations of the various pharmacokinetic parameters are shown in Table 7. It can be seen that CsA and M-17 administered at the same dose had similar pharmacokinetic parameters. There was no significant difference between $T_{\frac{1}{2}}$, the volume of distribution, or the clearance.

To determine whether M-17 was further metabolized to other CsA metabolites, the blood, urine, and bile obtained from the rabbits administered M-17 were analyzed for the presence of CsA metabolites. Representative chromatograms of these fluids are shown in Figure 14. In blood, only M-17 and M-18 were observed. In contrast, in bile and urine, other CsA metabolites, M-18, M-8, and M-26, were observed. The above results suggest and confirm the hypothesis that M-17 is a primary metabolite which is further biotransformed to other CsA metabolites.

DISCUSSION

CsA has a relatively narrow therapeutic index and there are large inter-individual differences in the response to a given dose. Thus, an understanding of the pharmacokinetics of the drug is essential in designing optimal therapeutic regimens. Further, very little is known with regards to the pharmacokinetic profile of CsA metabolites in any model system. Since tissue levels of CsA metabolites are higher than that of parent drug, it appears that CsA metabolites are sequestered

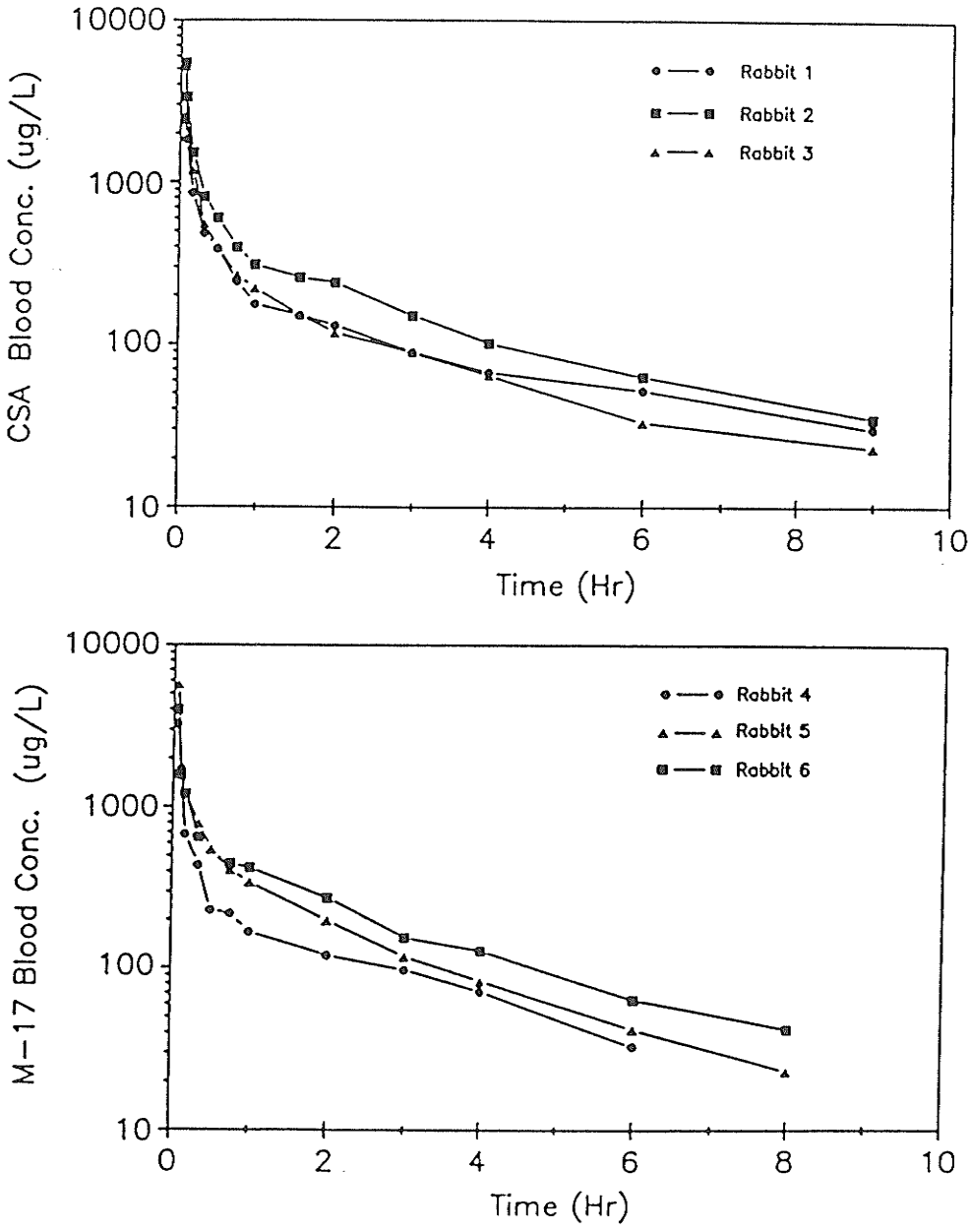

Figure 13. Pharmacokinetic profile for CsA and M-17 administered to rabbits. The pharmacokinetic profile for CsA (Top) and M-17 (Bottom) is demonstrated. Three separate rabbits were used for each drug.

Table 7. Pharmacokinetic parameters of CsA and its metabolite M-17 in rabbits.

	Rabbit number	Cl (mL/min/kg)	Vdss (L/kg)	$T_{\frac{1}{2}}$ terminal (hr)
CsA[a]	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
M-17[a]	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] CsA and M-17 were administered at a dose of 1 mg/kg i.v. over a period of three minutes.

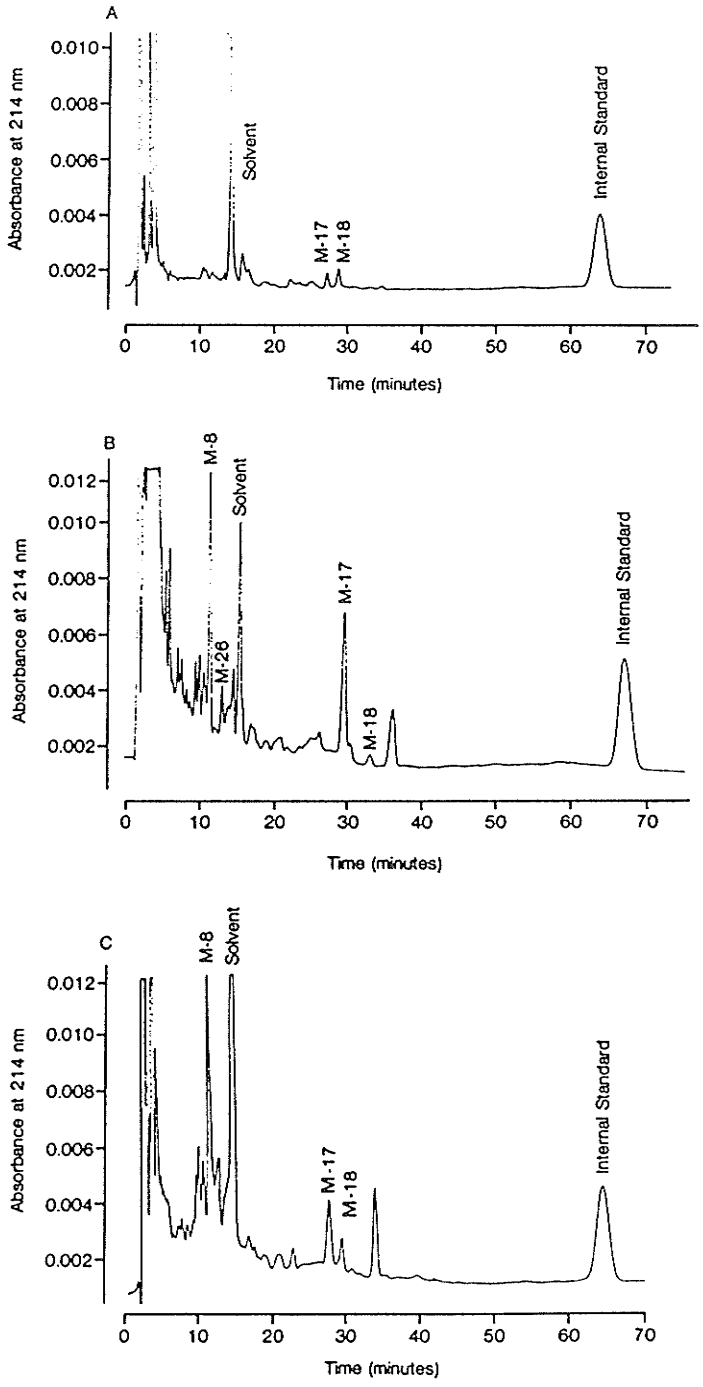

Figure 14. Representative HPLC chromatograms of blood, urine, and bile. Blood (A), urine (B), and bile (C) were obtained at time of sacrifice for rabbits administered M-17.

into tissues (159,160). Thus these increased tissue concentrations may be responsible for the adverse toxic effects. It is not known whether these higher tissue metabolite concentrations are due to pharmacokinetic differences, such as differences in volume of distribution (a measure of the extent of tissue binding) or the in situ metabolism of the drug by tissues.

Studies investigating CsA metabolite pharmacokinetics in humans have involved the administration of parent drug rather than a particular metabolite (168,206-208). Due to the complex kinetic relationship between the parent drug and its metabolites, the spectrum and accuracy of the metabolite pharmacokinetic parameters determined using this approach are limited (209). The administration of pure metabolite would alleviate these problems. Due to the lack of sufficient quantities of CsA metabolites, few studies in which individual metabolites have been administered have been performed. To date the only studies undertaken have involved the administration of M-17 to rats (150,157,158).

The hydroxylated CsA metabolite, M-17, is thought to be one of the primary metabolites of the parent compound (110,116). In human renal transplant recipients receiving CsA, M-17 is present at concentrations in blood and tissues in excess of CsA and most other identified metabolites (116,122,141,144). The subsequent metabolism of M-17 is thought to give rise to several other secondary metabolites; however, this hypothesis is based on the chemical structures of the metabolites rather than direct proof. In spite of the potentially important role of M-17, information regarding its

pharmacokinetics is limited (168,205,207).

Because of the deficiencies in previous studies, we undertook studies to determine the pharmacokinetics of M-17 and CsA after administration of a single i.v. dose. Using this approach, CsA and M-17 were found to have a similar volume of distribution, half-life, and clearance. Preliminary studies with CsA demonstrated a dose-dependent increase in the volume of distribution and the terminal half-life (data not shown). This is consistent with observations by Awni and Sawchuk (209), who also demonstrated a dose-dependent increase in elimination half-life and volume of distribution in rabbits administered CsA. It is therefore important that both parent drug and metabolite be administered at the same dosage for valid comparison of the pharmacokinetic parameters determined.

The similarities between the pharmacokinetic parameters of M-17 and CsA suggest that both compounds are cleared from the body at the same rate. The volume of distribution of CsA and M-17 were the same, thus suggesting that both drugs are bound to tissue to the same degree. The significance of such a finding, in light of the fact that M-17 is found in tissues at levels significantly higher than CsA (169), suggests that CsA may be metabolized in these tissues. The presence of intrinsic cytochrome P-450 activity in these tissues may result in the metabolism of CsA, thus producing increased metabolite levels. The significance of elevated metabolite tissue levels in the development of nephrotoxicity is not completely understood at this time.

CsA pharmacokinetics have been studied in a number of animal

models, including man (111,127,141,144). However, the majority of studies have employed the rat. This animal model is not ideal for the investigation of CsA pharmacokinetics due to the existence of species differences in the metabolism of the drug between the rat and man. M-1 is the primary CsA metabolite observed in rats (160), whereas in man and rabbits, M-17 is the major metabolite observed (111). This suggests a difference in the biotransformation and pharmacokinetics among different species. CsA pharmacokinetics in rabbits and man are similar (141,144). In addition, no significant differences have been detected in the structure or profile of metabolites obtained from either species.

Ryffel et al (150,158), using the rat model have shown that, after i.v. administration of M-17, the clearance of this metabolite was greater than that of CsA, although detailed pharmacokinetic calculations were not performed. The plasma peak levels of M-17 were about 8 fold lower than those of CsA and the half-life was significantly shorter than that for CsA. These results are in contrast to the results presented here and may be due to a number of factors. First, the rat handles CsA differently than the rabbit or man. Secondly, plasma levels of the drug and metabolite were measured. Since M-17 is sequestered by erythrocytes, lower levels may be present in the plasma than in whole blood. Thirdly, since M-17 was measured by a polyclonal antibody for CsA, other metabolites produced would also cross-react with the antibody. Interpretation of such results is difficult. Subsequent studies by Donatsch et al (157) examined the effect of CsA or M-17 administered subcutaneously at 50

mg/kg/day in rats. The C_{max} was 16 fold higher for CsA and the trough levels 36 fold higher despite the fact that the same dose was used for each compound.

The proposed pathways for the biotransformation of CsA are based on the structures of isolated metabolites (110,116). No direct evidence for the biotransformation has been reported. It is proposed that CsA is initially metabolized to three primary metabolites: M-1, M-17, and M-21. These so-called primary metabolites then undergo subsequent transformation giving rise to secondary metabolites. In our study, M-17 was demonstrated to be further metabolized to M-18, which results from the intramolecular cyclization of M-17, as well as M-8 and M-26, which are dihydroxylated products of M-17 and M-18, respectively (Figure 15). Additional metabolites, for example those with a short half-life or those produced at a low concentration, might arise from M-17 but might not have been detected at the time specimens were taken (12 hours post dose). The peak eluting after M-18, which was found in urine and bile, was not identified. The relative retention time of this metabolite was not consistent with any of the pure metabolites available as standards in our laboratory. The identity of this metabolite is consistent with M-25 according to the relative retention time data reported by Maurer et al (110). Since M-25 could arise from demethylation of M-17, this would support the biotransformation scheme proposed in Figure 13. The results presented in this thesis are the first to provide evidence for the subsequent metabolism of M-17 (a putative primary metabolite) to other secondary metabolites, thus supporting the proposed biotransformation pathways

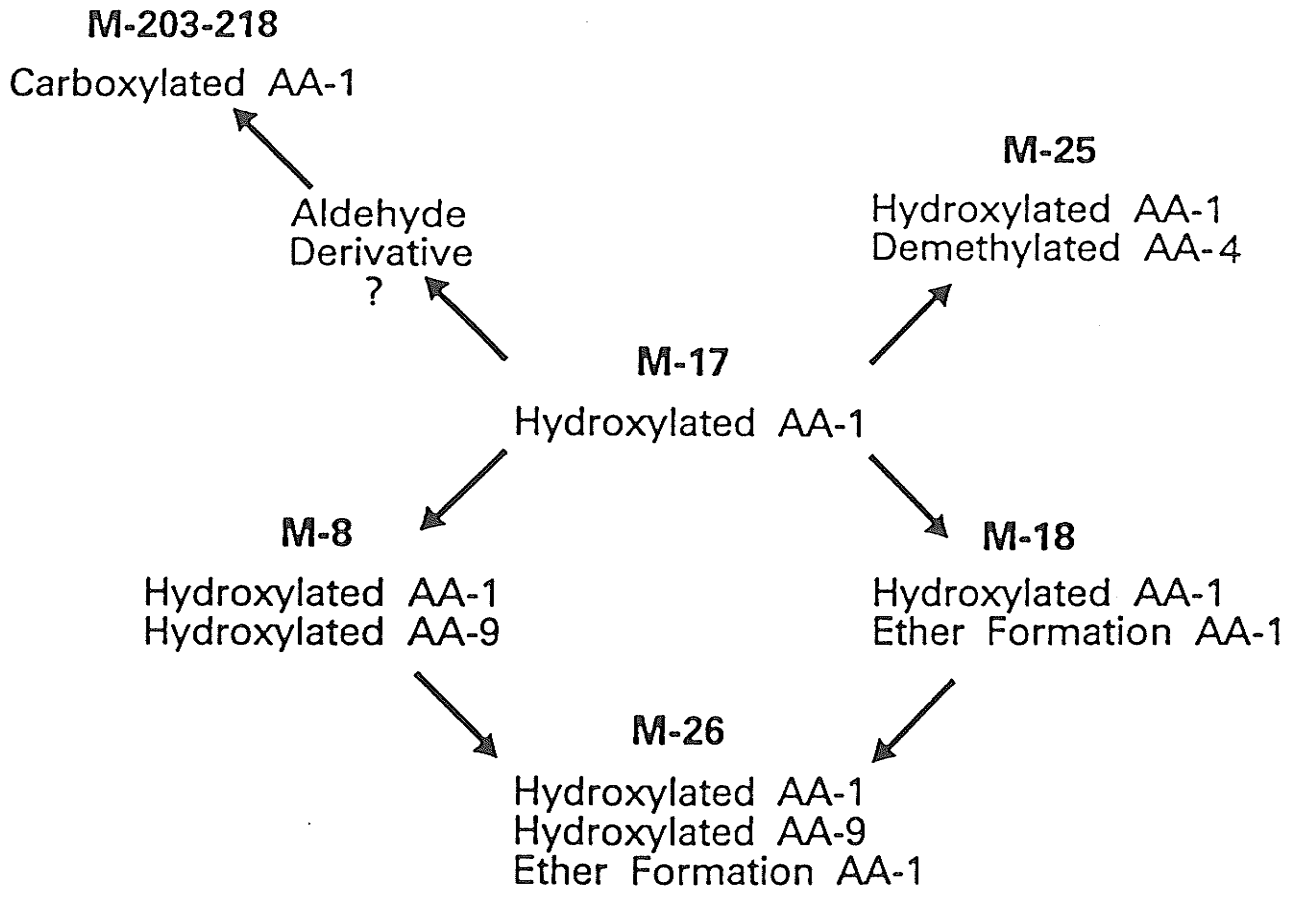

Figure 15. Proposed biotransformation pathways for the CsA metabolite M-17.

of CsA.

VI. CHAPTER 4

IN VITRO TOXICITY OF CYCLOSPORINE METABOLITES

METHODS

A. CULTURE OF LLC-PK_1 CELLS

Porcine tubular cells (LLC-PK_1) obtained from the American Type Culture Collection (Rockville, MD, USA) were grown in Medium-199 supplemented with non-essential amino acids, Earle's basic salts, and 5% fetal bovine serum (all from Gibco Laboratories, Burlington, ON). Cultures were plated at a density of 2×10^4 cells per 9 cm^2 tissue culture growth flasks. CsA, CsA metabolites, or vehicle (ethanol/Tween-80; 70/30 v/v) (final concentration of ethanol 0.2% v/v) were added at concentrations from 500 to 25,000 µg/L. Five days after plating, the cells were recovered by treatment with trypsin (0.05% w/v) and EDTA (0.05% w/v) and washed with media. Cell number was determined by counting viable cells (as measured by trypan blue exclusion) with a hemacytometer. A fraction of cell material was analyzed for protein content by the method of Lowry et al (211).

B. MEASUREMENT OF DNA, RNA, AND PROTEIN SYNTHESIS

DNA, RNA, and protein synthesis were determined as follows. The incorporation of [^{3}H]-thymidine (for DNA synthesis) into trichloroacetic acid (TCA) insoluble macromolecules in control, CsA, and metabolite-treated cells was determined. The metabolites examined were M-1, M-8, M-13, M-17, M-18, M-21, and M-26. Two µCi

[^{3}H]-thymidine (70 to 85 Ci/mmol, Amersham Canada Ltd., Oakville, ON) was added to the cell cultures 18 hours prior to trypsinization. For RNA and protein synthesis, 2 μCi of [^{3}H]-uridine (40 to 60 Ci/mmol) or 2 μCi of [^{35}S]-methionine (>800 μCi/mmol, Amersham Canada Ltd., Oakville, ON), respectively, was added to the cells. Subsequently, the cells were washed twice with saline and recovered by trypsin treatment as described above. The number of cells in each test well was determined by counting the viable cells as described above. The cells were lysed by freeze-thawing and the cellular material precipitated using 10% w/v TCA. The precipitate was resuspended in 1 mL saline and an aliquot (0.5 mL) removed for the determination of protein by the method of Lowry et al (211). The remaining volume (0.5 mL) was transferred to a sampling manifold filter apparatus (Millipore Corporation, Bedford, MA, USA) fitted with Whatman GF/C glass fibre filters. The filters were washed with 10 mL of 50% v/v ethanol. The radioactivity on the filter was determined by liquid scintillation counting using a quench corrected counting program on an LKB Rack Beta liquid scintillation spectrophotometer (LKB-Praudukete AB, Bramme, Sweden). All values were corrected for protein concentration. The 50% inhibitory concentration (IC_{50}) for CsA and each metabolite was determined. The potency ratio of each metabolite with respect to CsA was calculated using the following equation: IC_{50} of CsA/IC_{50} of metabolite. Each experiment was done in duplicate and repeated on three different occasions.

C. MORPHOLOGICAL INVESTIGATION OF LLC-PK_1 CELLS

Trypsinized cell suspensions grown in various concentrations of CsA, M-17, or M-21 were centrifuged at 300 xg for 10 minutes. The cell pellets were fixed for 1 hour at room temperature in 3% v/v glutaraldehyde in 0.1 M Sorenson's phosphate buffer, pH = 7.4. Subsequent to washing in the above buffer containing 0.2 M sucrose, cells were post fixed at room temperature for 1 hour in 1% osmium tetroxide in 0.1 M phosphate buffer. The samples were then processed for electron microscopy using standard procedures by Dr. J. Thliveris (Department of Anatomy, University of Manitoba).

D. ISOLATION OF RABBIT MESANGIAL CELLS

Primary cultures of mesangial cells were obtained from outgrowths of collagenase-treated isolated rabbit renal glomeruli as described below. Intact glomeruli were isolated from rabbit kidneys by modification of a procedure used for the isolation of glomeruli from rat kidney (212,213). Wherever possible aseptic techniques were used. Briefly, the kidneys from a New Zealand white rabbit (2.5 kg) were removed and placed in sterile Hank's balanced salt solution (HBSS) containing 25 mM Hepes, 100 U/mL penicillin, and 10 µg/mL streptomycin (all from Gibco Laboratories). The kidney was sliced into thin sections (1 to 3 mm) and the cortex removed with forceps and minced with a razor blade. The glomeruli were isolated from the homogenate by passing it through a series of sieves with decreasing pore size. The homogenate was passed through a 250 µm mesh screen and washed with HBSS. The supernatant was centrifuged, the pellet passed through a

150 μm and subsequently a 88 μm screen, and washed as described above. The glomeruli were collected on the 88 μm mesh and washed with HBSS. Microscopic examination revealed that the glomeruli were >95% free of tubule contamination.

The isolated glomeruli were incubated with trypsin (0.2% w/v) for 20 minutes at 37°C followed by incubation with 0.1% (w/v) Type IV collagenase (Sigma Chemical Co, St. Louis, MO, USA) (214 to 216). This procedure loosened up the glomeruli but gave few single cells. The collagenase-treated glomeruli were washed once with HBSS, resuspended in medium, and plated in 6 cm^2 tissue culture dishes. The growth medium used was Dulbecco's modified Eagle's (DMEM) supplemented with 20% heat-inactivated fetal calf serum, 100 U/mL penicillin, 10 μg/mL streptomycin, 1% non-essential amino acids (all from Gibco Laboratories), and 0.66 U/mL protamine-zinc insulin (Novo Laboratories Ltd., Willowdale, ON). After 10 days, subclones of individual cell outgrowths were removed. Using cloning rings, the cells were treated with a drop of trypsin (0.05% w/v) and EDTA (0.02% w/v), removed, and replated.

To avoid fibroblast contamination, the cells were grown for three passages in media in which L-valine was replaced by D-valine (214). Fibroblasts are unable to proliferate in D-valine-containing media since they lack the enzyme D-amino acid oxidase required for its utilization (217). The cells were subsequently passed every seven days in media devoid of antibiotics, since previous investigators have demonstrated a synergistic toxic effect between CsA and streptomycin (65). After 6 passages the cells were used for the subsequent

experiments described below.

Microscopic examination of the isolated cells revealed a homogeneous population of spindle shaped cells, with the absence of polygonal shaped cells (endothelial or epithelial cells). Morphologic examination of the isolated cells revealed ultrastructural features consistent with those of mesangial cells in vivo.

E. EFFECT OF CsA AND METABOLITES ON RABBIT MESANGIAL CELLS

The effect of CsA and metabolites on the isolated rabbit mesangial cells was examined using a modification of the protocol described above for LLC-PK_1 cells. Briefly, mesangial cells were plated at a density of 2 x 10^4 cells per 9 cm^2 six well tissue culture plates. CsA, metabolite (M-1, M-8, M-13, M-17, M-18, M-21, and M-26), or vehicle (ethanol/Tween-80; 70/30 v/v) were added to the cells at concentrations up to 25,000 µg/L (the final concentration of ethanol in each well was 0.2% v/v). The cells reached confluency after 5 days, at which time they were recovered by trypsin treatment, washed with media, and counted. A fraction was analyzed for protein content by the method of Lowry et al (211). DNA synthesis was determined as previously described for LLC-PK_1 cells above by measuring the incorporation of 2 µCi [^{3}H]-thymidine (70 to 85 Ci/mmol, Amersham Canada Ltd.) into TCA insoluble macromolecules 18 hours prior to trypsinization. Each experiment was done in duplicate and repeated three times for each concentration of drug or metabolite used. All values were corrected for protein concentration and the results expressed as the 50% inhibitory concentration (IC_{50}) for CsA and each

metabolite. The potency ratio of each metabolite with respect to CsA was calculated.

F. DETERMINATION OF PROSTACYCLIN RELEASE FROM RABBIT MESANGIAL CELLS

The release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable metabolite of prostacyclin (PGI_2), from mesangial cells was measured in the absence or the presence of CsA or metabolites using a modification of the procedure of Voss et al (76). These authors demonstrated a direct time and concentration effect of CsA on the release of prostacyclin from cultured endothelial cells using this protocol. CsA at 1,000 µg/L resulted in a significant reduction in prostacyclin release (76). Briefly, mesangial cells were grown to confluence in 9 cm^2 six well tissue culture plates. The medium was removed and the cells washed twice with serum-free DMEM. CsA, metabolite (M-1, M-8, M-17, M-18, M-21, and M-26), or vehicle control was added to the cells. CsA and metabolites were prepared in DMEM containing 0.5% fetal bovine serum. The final ethanol/Tween 80 (70:30; v/v) concentration was 0.2%. The effect of CsA and metabolites were examined at 500 µg/L and 5,000 µg/L. These concentrations were chosen for a number of reasons. CsA at 500 µg/L had minimal effects on cell function as previously reported. Further, this concentration is near the therapeutic range for blood levels of CsA in transplant recipients. CsA at 5,000 µg/L was near the IC_{50} value for cell cytotoxicity in the mesangial cells (a toxic concentration). The cells were incubated for 16 hours, the medium

removed, the cells washed twice with serum-free DMEM, and then incubated in 2 mL of medium for 15 minutes at 37°C. Subsequently, the media was removed, stored at 4°C, and analyzed for 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin within 24 hours using a commercially available RIA (Amersham Canada Ltd.). The cells were trypsinized and the protein content determined as previously described. The results were expressed as pmol 6-keto-$PGF_{1\alpha}$ released/15 minutes/mg protein. All experiments were repeated on at least three occasions. All values were expressed as a percent of control and expressed as mean $\pm$ SD. Comparison between the means of the groups was done using Student's t-test. A p value of 0.05 was considered significant.

G. DETERMINATION OF ENDOTHELIN RELEASE FROM ISOLATED RABBIT CELLS

Endothelin release from cultured mesangial cells was determined using a modification of the procedure previously reported by Nakahama (218). Using this method, a dose- and concentration-dependent increase in endothelin release from $LLC\text{-}PK_1$ cells treated with CsA was observed. The collection of supernatants within 4 hours resulted in minimal loss of cell integrity, as measured by cell viability, at CsA concentrations up to 10,000 µg/L. Briefly, the medium was removed from confluent mesangial cells grown in 9 cm^2 six well tissue culture plates. The cells were washed with sterile saline and twice with serum-free DMEM. CsA, metabolite, or vehicle control was added to the washed cells and incubated for 4 hours at 37°C. The effect of CsA and metabolites was examined at 500 µg/L and 5,000 µg/L using the stock

solutions prepared for prostacyclin release. Subsequently, the medium was removed and stored at 4°C (<24 hours). The concentration of endothelin was determined in the collected supernatants by RIA (Amersham Canada Ltd.). The protein content of the cells was determined as previously described. The final results were expressed as fmol endothelin/mg protein. All experiments were repeated on at least three separate occasions. All values were expressed as a percent of control and expressed as mean ± SD. Comparison between the means of the groups was done using Student's t-test. A p value of <0.05 was considered significant.

RESULTS

A. EFFECT OF CsA AND METABOLITES ON LLC-PK_1 CELLS (219)

The effect of CsA and metabolites on the growth of LLC-PK_1 cells as measured by cell viability is shown in Figure 16. CsA was much more toxic to the cells than any of the metabolites examined. CsA at concentrations <2,000 µg/L significantly inhibited cell growth. In contrast, most metabolites even at concentrations as high as 25,000 µg/L had little effect. The exception was M-21, which totally inhibited cell growth, and M-17 and dihydro-M-17, which inhibited growth by 50%. The effect of CsA and metabolites on the various metabolic parameters is summarized in Table 8. The results are represented as the concentration required for 50% inhibition of the particular metabolic function. As with DNA synthesis, no metabolite was as effective as CsA in inhibiting RNA or protein synthesis.

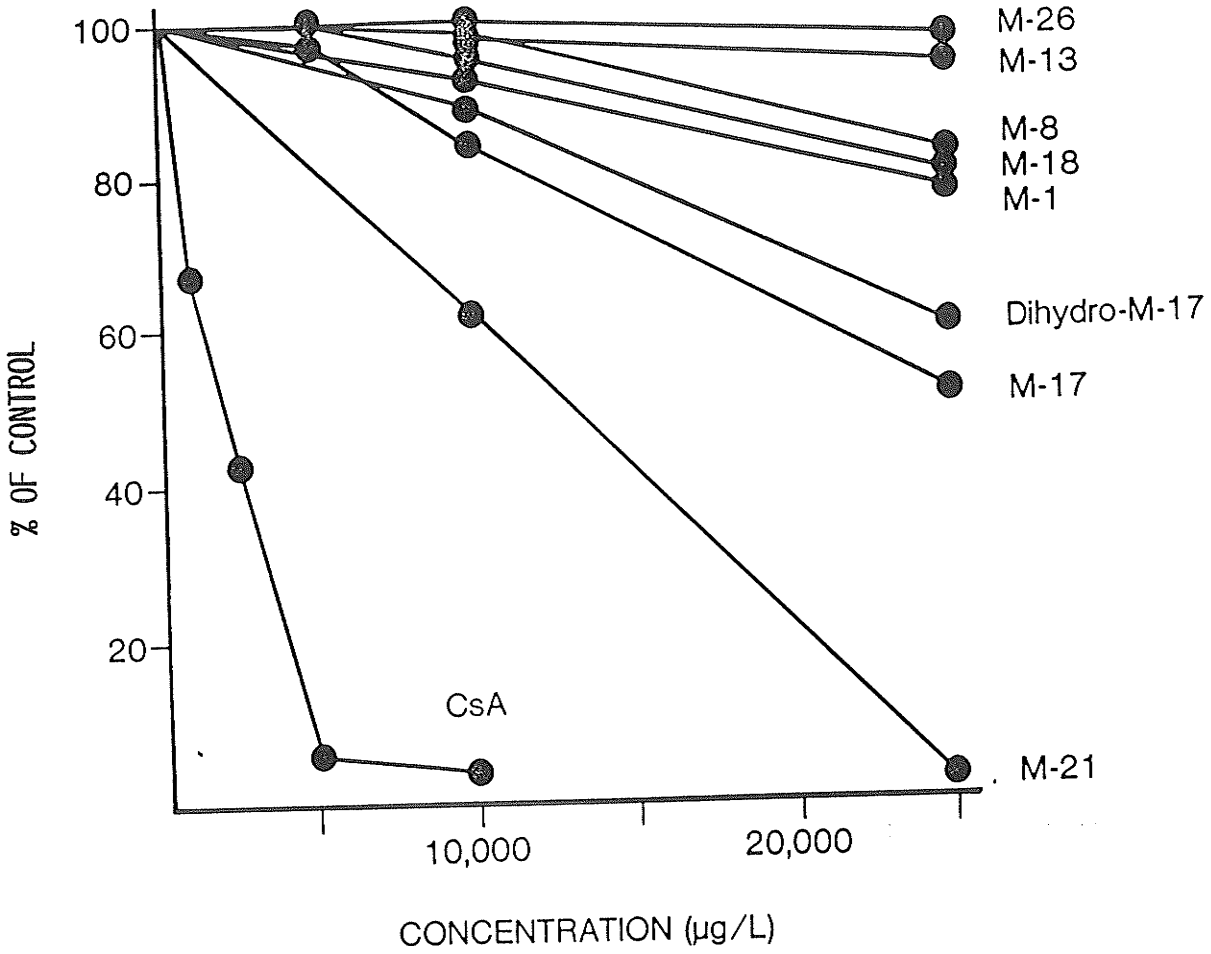

Figure 16. Effect of CsA and CsA metabolites on the growth of LLC-PK$_1$ cells. The growth of LLC-PK$_1$ cells in the presence of increasing concentration of CsA and metabolites is listed. Cells were grown in the absence or presence of drug or metabolite for 6 days. The results are represented as a mean percent inhibition of growth (duplicate experiments run on separate days) as compared to growth of control cultures. Values from duplicate experiments varied <10%.

Table 8. Comparison of the effects of metabolites on metabolic parameters in LLC-PK$_1$ cells.[a]

Metabolite	Cell growth	DNA synthesis	RNA synthesis	Protein synthesis
CsA	2,000 (1.00)[b]	2,500 (1.00)	6,000 (1.00)	3,800 (1.00)
M-17	25,000 (0.08)	>25,000 (<0.10)	>25,000 (<0.24)	>25,000 (<0.15)
M-21	11,000 (0.18)	15,000 (0.16)	12,000 (0.50)	12,000 (0.32)
M-1, M-8, M-26, M-13, M-18 Hydroxylated, Saturated Metabolite	>25,000 (<0.08)	>25,000 (<0.10)	>25,000 (<0.24)	>25,000 (<0.15)

[a] Results expressed as the concentration of metabolite (μg/L) required for 50% inhibition of function.

[b] Results in parentheses indicate potency relative to CsA (CsA = 1.0).

However, M-21 exhibited the greatest inhibitory effect with potency ratios of 0.17 to 0.50 of that of CsA for the metabolic parameters examined. The effect of CsA metabolites on the various parameters at the maximum concentration tested (25,000 μg/L) is shown in Figure 17. At this concentration CsA completely inhibited cell growth, DNA, RNA, and protein synthesis. The majority of metabolites, including M-17, the major CsA metabolite, resulted in less than 25% inhibition of these parameters. The exception were M-17 and dihydro M-17 which resulted in a 40 to 50% inhibition of cell growth, and M-21 which resulted in greater than 90% inhibition of all parameters.

Cell growth in the presence of 2,500 μg/L CsA or 10,000 μg/L M-21 for 6 days resulted in an increased number of vacuoles as compared to controls (Figure 18). These morphological alterations are similar to what is observed in CsA tubular toxicity in man (31-34). No such morphological changes were seen for cells grown in the presence of M-17 up to the highest concentration tested (25,000 μg/L). Only these two metabolites were assessed since M-21 revealed the most toxic effect other than CsA. Moreover, M-17 was viewed as representative of the remaining metabolites since they, as well as M-17, had similar or less potencies in terms of their toxic effects of the various metabolic parameters studied.

B. EFFECT OF CsA METABOLITES ON RABBIT MESANGIAL CELLS

Table 9 summarizes the cytotoxic effects of CsA metabolites on the isolated rabbit mesangial cells. The degree of cytotoxicity was determined by measuring cell number and DNA synthesis in the presence

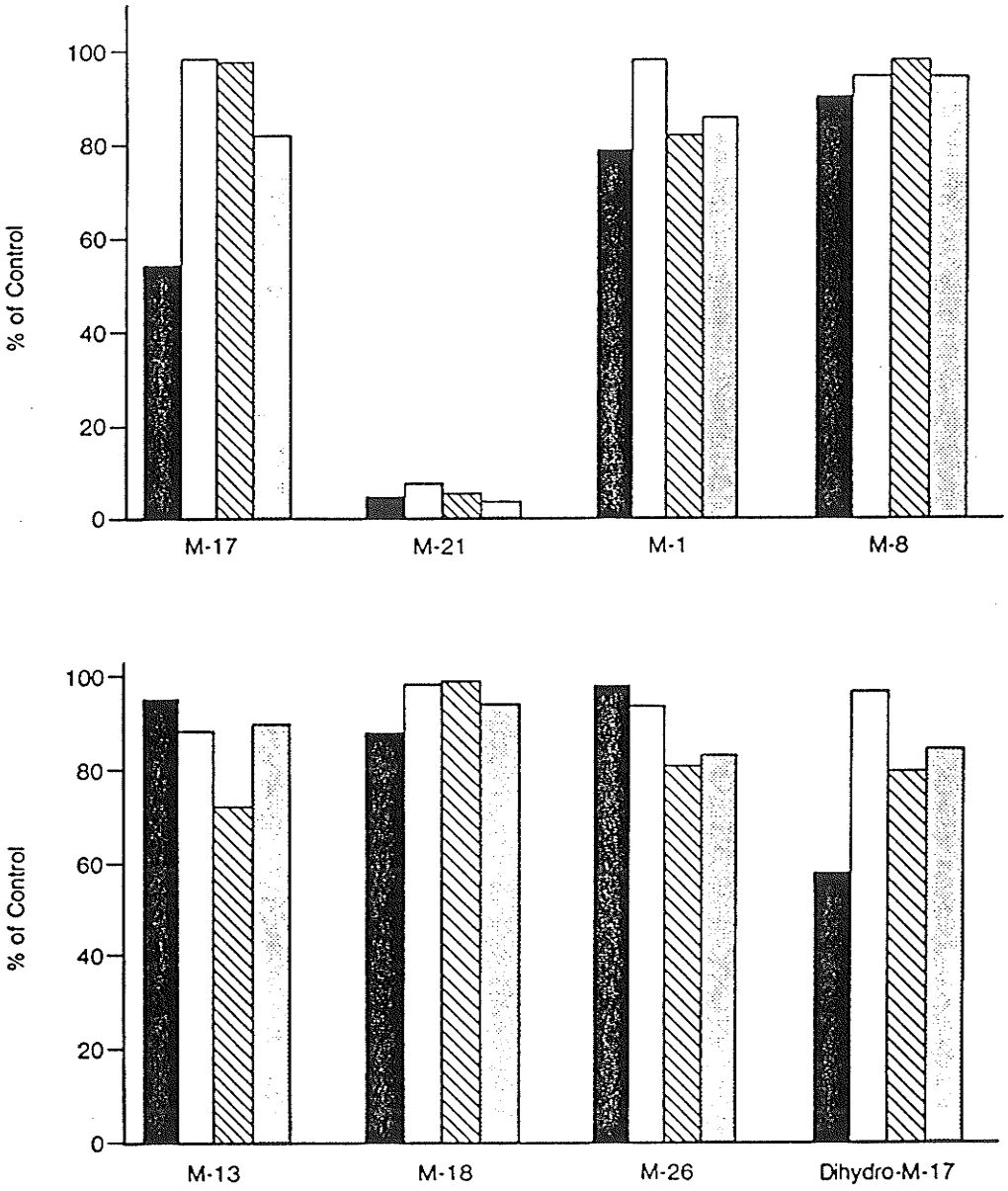

Figure 17. The effect of CsA metabolites on various growth parameters of LLC-PKC$_1$ cells. The effect of several CsA metabolites at 25,000 µg/L on cell growth (■), DNA (□), RNA (▨), and protein synthesis (▩) was examined. Results are expressed as a mean % inhibition (duplicate experiments) of the various metabolic parameters examined as compared to control cultures. Values from duplicate experiments varied <10%.

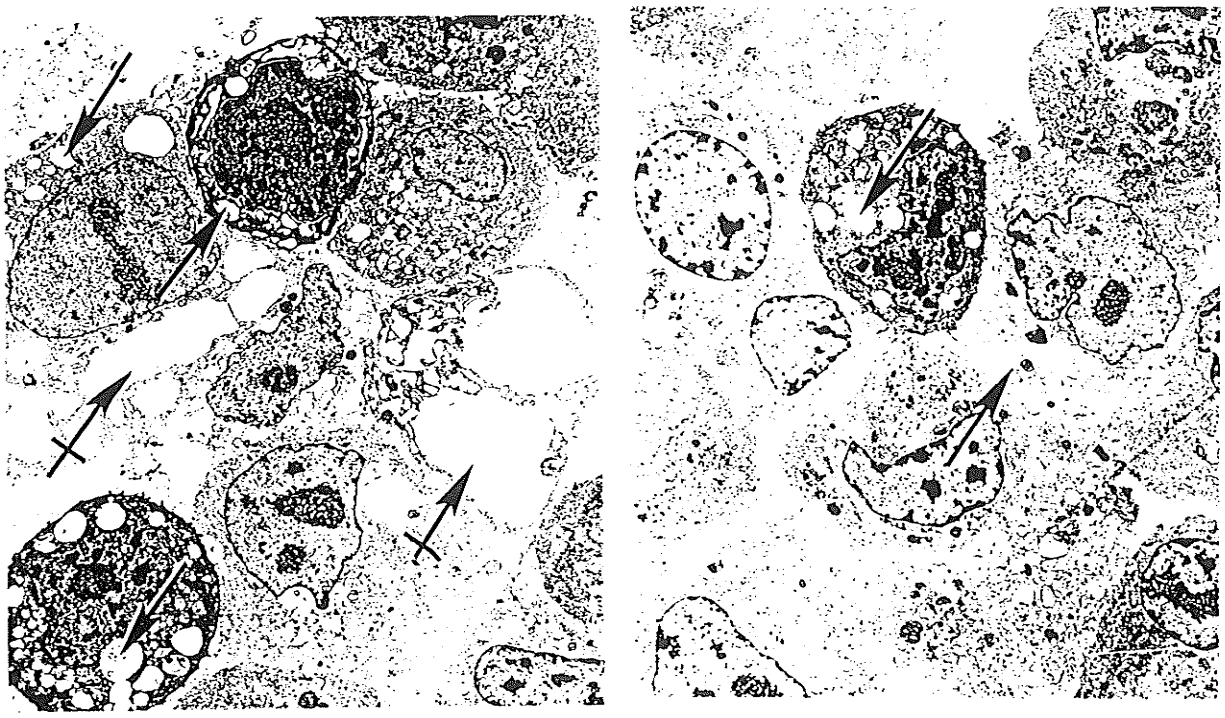

Figure 18. Morphological assessment of CsA metabolite toxicity of LLC-PK_1 cells. Left. Renal epithelial cells (LLC-PK_1) cultured for 5 days in 2,500 µg/L CsA. Note numerous vacuoles (arrows) which were frequently enlarged (crossed arrows). Right. Control renal epithelial cells (LLC-PK_1) cultured for 5 days. Cellular integrity is well maintained during culture period. Note the moderate number of vacuoles (arrows). Magnification x 2,800.

Table 9. Comparison of the effects of CsA metabolites on metabolic parameters in rabbit mesangial cells.[a]

Metabolite	Cell growth		DNA synthesis	
CsA	5080 ± 560	(1.00)[b]	7000 ± 670	(1.00)
M-1	18,000 ± 750	(0.28)	18,200 ± 1250	(0.38)
M-8	>25,000	(<0.20)	>25,000	(<0.28)
M-17	25,000 ± 4800	(0.20)	>25,000	(<0.28)
M-18	>25,000	(<0.20)	>25,000	(<0.28)
M-21	14,700 ± 600	(0.35)	12,600 ± 600	(0.56)
M-26	>25,000	(<0.20)	>25,000	(<0.28)

[a] Results expressed as the concentration of metabolite (µg/L) required for 50% inhibition of function (n = 3).

[b] Results in parentheses indicate potency relative to CsA.

of metabolites. Previous studies with the LLC-PK_1 cells determined that the results obtained for inhibition of protein and RNA synthesis by CsA and metabolites paralleled those obtained for DNA synthesis, thus only this latter parameter along with cell growth was determined. M-21 was the most active metabolite examined having potencies relative to CsA of 0.35 for inhibition of cell growth and 0.56 for inhibition of DNA synthesis. All other metabolites were found to exhibit minimal effects at the concentrations examined. In all cases the other metabolites (M-17, M-13, M-1, M-18, M-26, and M-8) were <20% as effective as CsA with respect to cell growth and <28% as effective with respect to DNA synthesis. Figure 19 compares the effect on DNA synthesis at both 10,000 µg/L and 25,000 µg/L (the two highest concentrations examined). The results obtained with the rabbit mesangial cells are similar to those obtained with the LLC-PK_1 cells, thus confirming the nephrotoxic properties of the CsA metabolites previously determined using these cells.

C. EFFECT OF CsA METABOLITES ON THE RELEASE OF PROSTACYCLIN AND ENDOTHELIN FROM RABBIT MESANGIAL CELLS

The effect of CsA and metabolites on the release of 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, was determined. A summary of the results is shown in Figure 20. CsA resulted in a dose-dependent reduction in prostacyclin production over control values. A similar reduction in prostacyclin production was observed for most CsA metabolites. In addition, CsA and CsA metabolites all exhibited a similar reduction over control values ($p<0.05$). Interestingly, in all

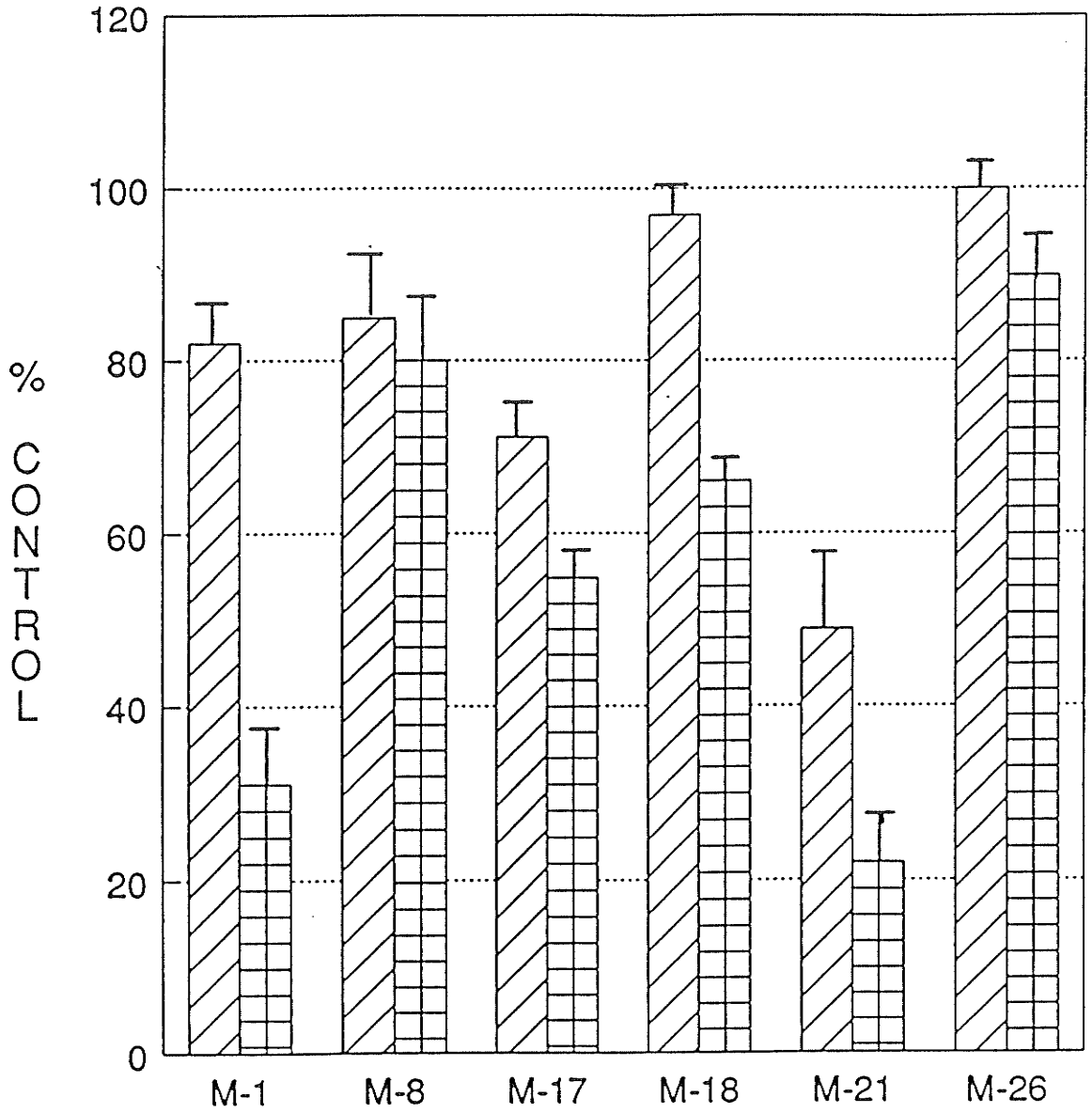

Figure 19. The effect of CsA metabolites on DNA synthesis of isolated rabbit mesangial cells. The effect of several CsA metabolites on DNA synthesis was examined at 10,000 µg/L (▨) and 25,000 µg/L (▦). Results are expressed as mean ± SD (n = 3) of the percent inhibition of DNA synthesis as compared to control cultures.

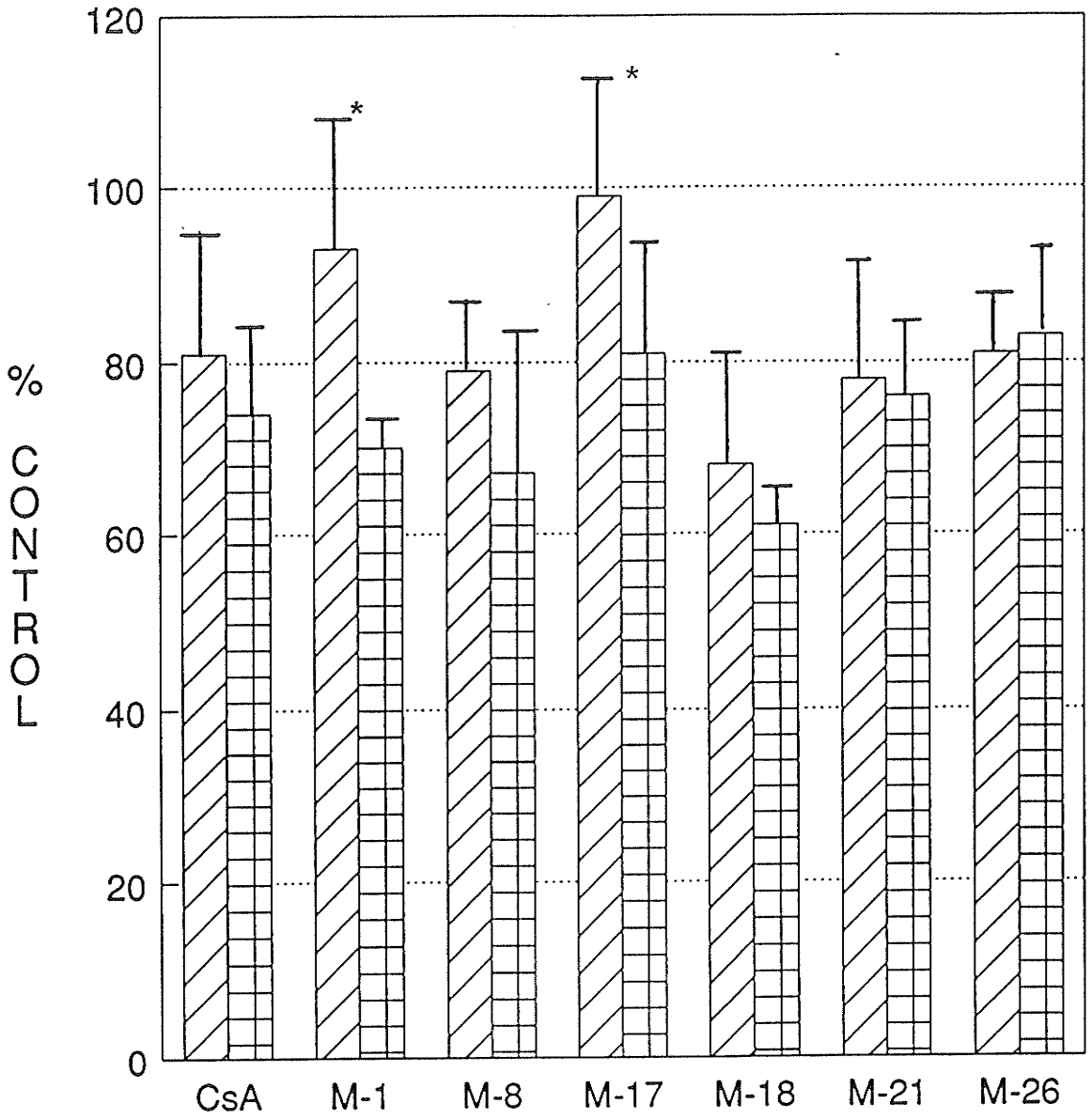

Figure 20. The effect of CsA and CsA metabolites on prostacyclin release from cultured rabbit mesangial cells. CsA and metabolites were examined at 500 µg/L (▨) and 5,000 µg/L (▦). The results are expressed as the mean ± SD (n = 3) of the mean percent change in prostacyclin release as compared to control cultures. *Not significant at $p<0.05$ with respect to control cultures.

cases CsA metabolites were equipotent as CsA, resulting in a 20 to 40% reduction in prostacyclin production.

The effect of CsA and metabolites on the release of endothelin is summarized in Figure 21. CsA at concentrations of 500 μg/L did not affect the release of endothelin from mesangial cells; however at 5,000 μg/L CsA resulted in a reduction in its release. The majority of metabolites did not affect the release of endothelin; however, M-8, M-17, and M-26 resulted in a significant increase ($p<0.05$) in production as compared to control values at concentrations of 5,000 μg/L.

DISCUSSION

Nephrotoxicity is the side-effect of CsA therapy that is the most serious and of most concern (12,20-34). The progression of CsA nephrotoxicity has been well documented; however, its precise mechanism is not known. Furthermore, the role of CsA metabolites in the development and progression of CsA nephrotoxicity has not been clearly elucidated. Clinical studies measuring both parent and metabolite concentrations have yielded only limited information concerning the potential significance of CsA metabolites in CsA nephrotoxicity. High circulating levels of primary metabolites in trough blood have been reported for most transplant types (122,130,131,140). Selective measurement of metabolite concentrations in clinical samples has been performed in several transplant centres in order to determine whether there is a correlation between

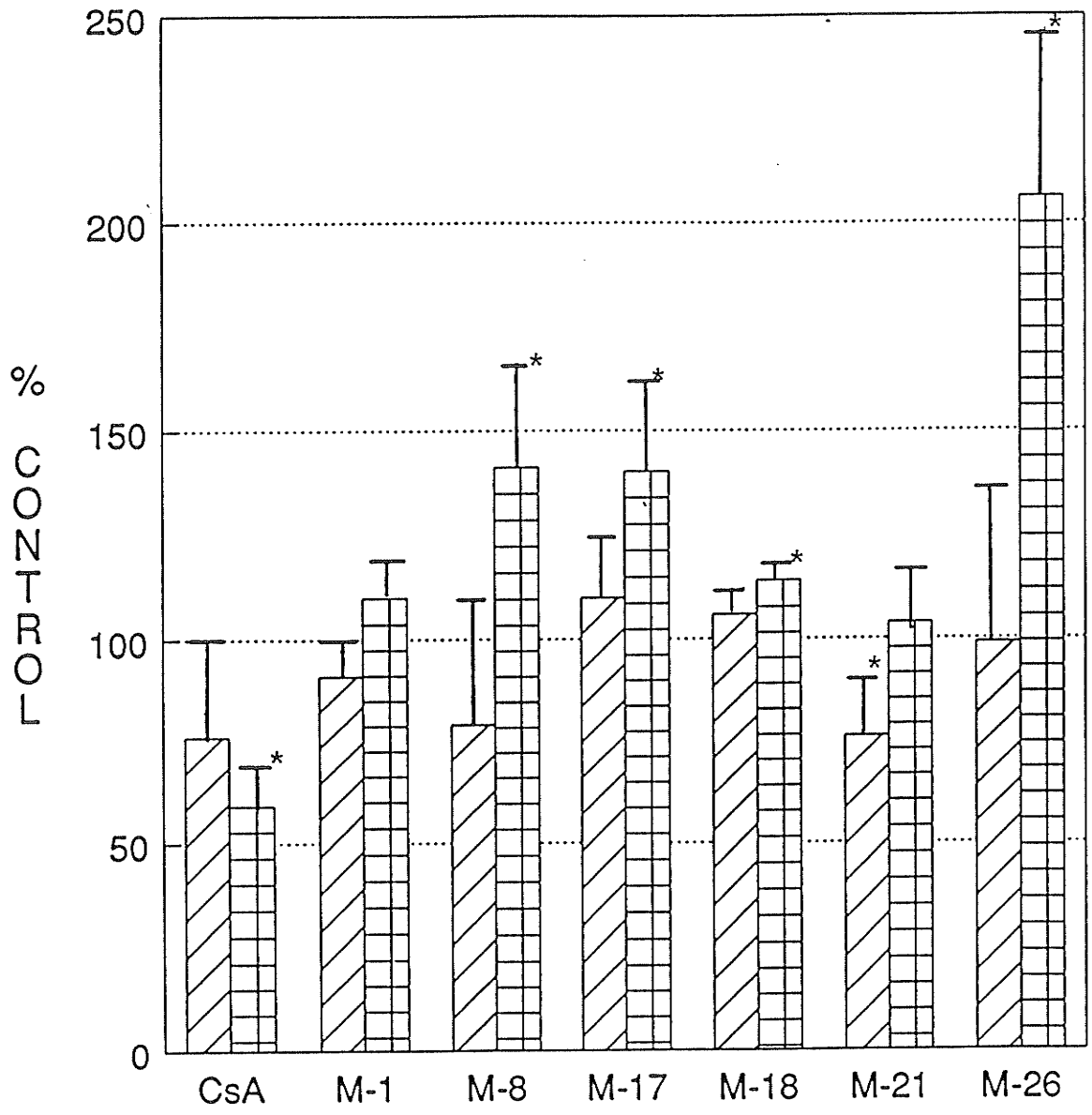

Figure 21. The effect of CsA and CsA metabolites on endothelin release from cultured rabbit mesangial cells. CsA and metabolites were examined at 500 µg/L (▨) and 5,000 µg/L (▦). The results are expressed as the mean ± SD (n = 3) of the mean percent change in endothelin release as compared to control cultures. *Significantly different from control cultures ($p<0.05$).

metabolite concentration and clinical events. There have been several studies which provide evidence for the importance of CsA metabolites in the development of CsA nephrotoxicity. Studies by Yee et al (170) and Wonigeit et al (171) provide evidence that not only the parent compound, but also some of its metabolites may exert nephrotoxic effects when present in high concentrations. In addition, Sewing et al (172) found a high correlation between nephrotoxicity and the dihydroxylated metabolites of CsA, particularly M-26. In contrast to these studies, other investigators have found that there was no additional predictive value in the correlation of metabolite concentrations to clinical outcome in renal allograft recipients (121). At this time the role of CsA metabolites in nephrotoxicity remains unclear due in part to the limited availability of CsA metabolites required for both in vitro and in vivo studies. The discovery of CsA metabolites that possess significant toxic properties would have important consequences with respect to the clinical management of patients receiving the drug.

CsA toxicity has been previously examined using a number of in vitro cell systems. The LLC-PK_1 cell line has been used by several investigators to examine the toxic effects of CsA (51,63-69). In addition, CsA toxicity has been studied using other cell types including mesangial (42,72,73), endothelial (74,75), hepatocyte (153,155), and epithelial cells (70,152). However, studies examining the potential toxic effects of CsA metabolites both in vitro and in vivo have been limited. Because of the difficulty in obtaining sufficient metabolite quantities, in vitro assay systems have a

potentially important role in assessing metabolite toxicity. Furthermore, because of the complex interaction between many different factors in CsA nephrotoxicity, dissection of the cell into individual cell types may help to elucidate the mechanism and precise site of damage to the kidney.

Initially, the potential toxicity of CsA metabolites was investigated using the LLC-PK_1 cell line. This cell line was initially derived from porcine kidney renal tubule epithelial cells. Renal tubular effects of CsA occur independently with respect to the development of CsA nephrotoxicity (13,32). These changes are of little clinical consequence. For this reason, subsequent studies involved the use of a primary cell line derived from isolated rabbit mesangial cells. However, since LLC-PK_1 cells have been the most frequently used cell line to investigate CsA nephrotoxicity, the effect of CsA metabolites on this cell line was investigated. The use of this cell line allows comparison to the data in the literature.

The rabbit mesangial cell was chosen for a number of reasons. Mesangial cells occupy a central role in the renal glomerulus (77). These cells are responsible for maintaining renal vasculature tone by contracting or relaxing in response to various stimuli released by neighboring mesangial cells or other cell types such as endothelial cells or platelets (77). Because of their potential importance in maintaining renal vascular tone, mesangial cells are thought to have a crucial role in the development and progression of CsA nephrotoxicity. The use of a primary cell line has several advantages over the use of immortalized cell lines such as the LLC-PK_1 cell line. Immortal cell

lines often undergo phenotypic changes and exhibit different susceptibility to various cell activators. The results obtained using such a cell line may be solely reflective of this phenotypic alteration. Finally, since the nephrotoxicity observed in the rabbit may be more indicative to that seen in man (62), the rabbit was chosen as the species from which to isolate the cells.

Using the LLC-PK_1 cells, we demonstrated that CsA metabolites were considerably less toxic than the parent drug when DNA, RNA, and protein synthesis were determined. M-21 was the most potent of all metabolites tested. At 25,000 µg/L it resulted in complete cell death and inhibition of DNA synthesis. At this concentration all other metabolites had minimal effects. In contrast CsA at concentrations as low as 2,500 µg/L exhibited significant toxic effects. Similarly, it was also demonstrated that the toxic effects of CsA metabolites on mesangial cells were significantly less than those observed for the parent drug. CsA metabolites were not nearly as effective as parent drug in inhibiting cell growth and DNA synthesis. The potencies of the metabolites with respect to CsA were similar to those obtained for the LLC-PK_1 cells. M-21 was the most effective metabolite in inhibiting these metabolic functions; M-21 was 35 and 56% as effective as CsA in inhibiting cell growth and DNA synthesis, respectively. In contrast to our results obtained with the LLC-PK_1 cells, M-1 exhibited potent toxic properties, albeit not to the same degree as CsA in the mesangial cell cultures. All other metabolites tested exhibited significantly less activity.

The results of this thesis are consistent with those reported by

Cole et al (151), who demonstrated that M-1 and M-17 were considerably less toxic than CsA in LLC-PK_1 cells. These metabolites at a concentration of 10,000 µg/L had minimal effects on DNA, RNA, or protein synthesis. In contrast, CsA at 500 µg/L resulted in significant inhibition of these metabolic functions. Metabolite toxicity has also been examined in other cell types. Wilson and Hreniuk (152) demonstrated toxic effects of CsA on cultures of renal epithelial cells isolated from micro-dissected individual tubules. Toxicity was assessed by nigrosine dye uptake. Bowers (155) studied the effect of five CsA metabolites on this cell system. M-1, M-18, and M-21 were the most toxic, whereas M-17 was moderately toxic. Unfortunately, the relative toxicity (potency) to CsA was not reported. In this same study, the effects of these metabolites on hepatocyte cultures was examined. Toxicity was determined by measuring the inhibition of taurocholate conjugation. M-17 was the most toxic metabolite, whereas M-18 and M-1 were less toxic; again no potency with respect to CsA was reported. To date, there have been no reports examining the effect of CsA metabolites on mesangial cells; however, the effect of CsA on this cell type has been well documented (42,71-73).

The morphological changes commonly observed in renal biopsies obtained from patients treated with CsA have been well documented (31-38). Of interest is the vacuolization of the proximal tubules, a derangement occurring due to CsA toxicity. LLC-PK_1 cells grown in the presence of CsA or M-21 at levels that were toxic to the cells contained vacuoles in the cell cytoplasm. The administration of M-17,

at concentrations which were not cytotoxic, resulted in no observable alterations. Thus the results observed in vitro parallel those obtained in vivo.

A comparison of the in vitro immunosuppressive activity and toxicity of CsA metabolites obtained suggests that the two may be dissociated. M-1, M-17, and M-21 have been shown to be the most immunosuppressive of all metabolites having activity in the range of 10 to 20% of CsA. In contrast M-21 was the most toxic metabolite followed by M-1; M-17 was substantially less toxic. In the above studies, M-21 was found to have a potency of up to 50% of CsA in inhibiting the metabolic parameters investigated to examine toxicity. Thus the order of immunosuppressive activity does not correlate with toxicity. This is similar to the findings of Bowers et al (155) who also reported a lack of correlation between immunosuppression and toxicity for CsA metabolites. This suggests that the region of the CsA molecule responsible for immunosuppression may be distinct from its toxic domain. This raises the possibility of finding an analogue of CsA that is not toxic, but still retains immunosuppressive properties.

The concentration of CsA required to inhibit the metabolic parameters of both mesangial and LLC-PK_1 cells are significantly higher than their respective concentrations found in vivo in the blood of transplant recipients. It has previously been shown that the tissue concentrations (kidney, liver, pancreas, and spleen) of both CsA and metabolites exceed those found in blood or plasma (130,131,142). The concentrations of drug and metabolites used in

these toxicity studies are therefore more consistent with those found in tissues than those of circulating blood levels. Since blood and tissue levels of metabolites often exceed those of the parent drug, high CsA metabolite concentrations have the potential to be of significance. However, the clinical findings of the toxic effect of M-21 is not clear. The concentration of this metabolite in plasma and tissues is quite low when compared to CsA and other metabolites, in particular M-17 (130,131,220). One could therefore assume that its role overall in vivo is most likely minimal. In contrast, the second most toxic metabolite, M-1, is found in significant amounts in both blood and tissue. The role of this metabolite in vivo remains to be determined.

The precise cause of CsA nephrotoxicity is not known. It is generally believed that functional changes to the arterioles and mesangium in the form of vasoconstriction are responsible for the renal dysfunction seen during drug therapy (32,33). One hypothesis for the pathogenesis of CsA nephrotoxicity involves the activation of vasoconstrictor prostanoids and other vasoconstrictor substances such as endothelin and/or inhibition of vasodilator substances, such as PGI_2 (prostacyclin) and PGE_2 (32,33). A critical balance between these vasoactive substances is required to maintain normal vascular tone. Thus, small increases in vasoconstrictive compounds coupled with small decreases in vasodilatory substances could lead to considerable increases in vasoconstriction of the renal vasculature. In view of the potential role of prostanoids such as prostacyclin and endothelin, further studies were undertaken to examine the acute

effects of CsA and metabolites on the release of these two potent vasoactive substances from isolated rabbit mesangial cells.

A concentration-dependent decrease in prostacyclin production, as measured by the release of its stable metabolite 6-keto-$PGF_{1\alpha}$, was observed in cells treated with CsA and CsA metabolites. CsA metabolites were as equipotent as CsA with regards to reducing the production of prostacyclin. Treatment of the cells with 5,000 µg/L CsA or metabolite resulted in a 20 to 40% reduction in its synthesis. The finding of a decreased production of prostacyclin (a vasodilator substance) by CsA suggests that both parent drug and its metabolites can lead to increased vasoconstriction of the renal vasculature subsequent to CsA administration. However, the extrapolation of these results to the in vivo situation where other factors can influence renal vasculature tone may not be applicable.

The effect of CsA on the release of prostacyclin seen here is similar to that reported by others for various other cell types. However, to date no study has examined the direct effects of CsA metabolites on its release. Voss et al examined the release of prostacyclin from cultured endothelial cells (76). CsA at 1,000 µg/L led to a 50% reduction in prostacyclin production. In addition, a dose-dependence reduction was observed. Similarly, Rosenthal (221) demonstrated a dose-dependence decrease in prostacyclin production; however, at 1,000 µg/L only a 10 to 15% reduction in production was observed. Besides prostacyclin, the effect of CsA on the release of other prostanoids has been studied. Stahl et al (49) demonstrated that CsA at concentrations as low as 1,600 µg/L could significantly

suppress PGE_2 formation. PGE_2, like prostacyclin, is a potent vasodilatory prostanoid. CsA-induced alteration in production of prostacyclin from various renal cells could have serious effects on vascular tone, ultimately resulting in increased vasoconstriction (32,33).

Previous investigators have postulated that CsA results in an increase in production of endothelin, a potent vasoconstrictor, in renal tissue (54). The implication of endothelin in the development of CsA nephrotoxicity comes from several sources. Infusion of endothelin into rats results in significant hypertension and long lasting vasoconstriction of the renal arterioles. In CsA-treated rats, endothelin levels were markedly higher than in control animals. Furthermore, infusion of an anti-endothelin antibody was able to revert the hypertensive effects of endothelin (decreased GFR and renal plasma flow). In the studies performed in this thesis, CsA was shown to have little effect on the release of endothelin from isolated rabbit mesangial cells. However, M-26 and M-8 were found to result in a significant increase in endothelin production by these cells.

These findings are in contrast to the data presented by Nakahama (218) who demonstrated a dose- and time-dependent increase in endothelin production from $LLC\text{-}PK_1$ cells treated with CsA. The reason for this discrepancy is not clear, however it could be due to cell and species differences. Voss et al (76) reported a cell and species difference in the production of prostacyclin by various cell types and species. However, the finding that certain CsA metabolites resulted in an increased production of endothelin suggests a possible role for

these metabolites in the development of nephrotoxicity. The finding of increased production of endothelin by M-26 and M-8 (both dihydroxylated) is consistent with the studies by Sewing et al (172) who demonstrated a high correlation between CsA nephrotoxicity in liver allograft recipients and the double hydroxylated metabolites of CsA, especially M-26, but not with parent CsA itself. This suggests a positive role for this metabolite in development of CsA nephrotoxicity.

In vivo studies investigating the toxic activities of CsA metabolites have been hampered by the lack of sufficient quantities of metabolites and a suitable animal model (62). Preliminary studies investigating the nephrotoxic properties of CsA metabolites in vivo have been reported (150,157-159). M-17, when administered i.p. to rats at a dose of 10 mg/kg/day for 28 days, resulted in no morphological or biochemical evidence of nephrotoxicity (150). In contrast, a similar dose of CsA resulted in a significant decrease in creatinine clearance and renal morphological changes consistent with CsA nephrotoxicity. Although the doses of CsA and M-17 were similar, the average concentration of M-17 in whole blood was less than half of that for CsA. In a recent study (157) in which the primary metabolites of CsA, AM1, AM9, and AM4N were administered at a dose of 50 mg/kg/day p.o. to SH rats for 28 days, no biochemical or morphological evidence of nephrotoxicity was observed. In contrast, CsA at this concentration resulted in biochemical and morphological alterations consistent with CsA nephrotoxicity. It should be noted that despite the same administered dose, the blood trough levels for

the metabolites were substantially less than those for CsA. In addition, the rat is not a suitable model for assessing nephrotoxicity since it has been shown that much higher concentrations of CsA are required for inducing renal changes in rats as compared to humans.

A recent study by Kim et al (222) examined the in vivo toxic effects of a mixture of CsA metabolites (consisting of 5 metabolites including M-17, M-18, and M-1) on hepato- and nephrotoxicity in a rat small intestine transplant model. CsA or the pool of five metabolites were administered to rats at a dose of 7.5 mg/kg/day s.c. for seven days. The CsA metabolite-treated group, in contrast to the CsA-treated group, demonstrated no apparent hepatic or nephrotoxicity. Surprisingly, no histologic alterations were observed in the CsA-treated animals. This is most likely due to the short period of administration of CsA. In addition, there were no appreciable levels of CsA metabolites detected in the metabolite-treated group despite the fact that the animals receiving parent CsA at a similar dose did. These authors suggest that this could be due to differences in the pharmacokinetics between CsA and its metabolites. This finding is consistent to that reported by Ryffel et al (157), who reported substantially lower blood levels of metabolites in metabolite-treated animals as opposed to the CsA-treated animals.

The role of CsA metabolites in toxicity remains unclear. The findings of this thesis suggest that CsA metabolites are less cytotoxic than the parent drug, at least in the in vitro models examined. The majority of metabolites exhibited minimal toxicity with the exception of M-21 and, to a lesser extent, M-1. However, some CsA

metabolites did result in increased release of endothelin and a decrease in production of prostacyclin from mesangial cells. These in vitro findings suggest that CsA metabolites can alter renal hemodynamics producing alterations consistent for CsA nephrotoxicity in vivo. However, further studies are required to positively confirm these findings.

VII. CHAPTER 5

THE ISOLATION, STRUCTURAL CHARACTERIZATION, AND IMMUNOSUPPRESSIVE ACTIVITY OF CYCLOSPORIN G (NVa^2-CYCLOSPORINE) METABOLITES

METHODS

A. ISOLATION OF CsG METABOLITES

Urine was obtained from normal subjects receiving a single oral dose (2.5 mg/kg) of the drug (Dr. H. Schran, Sandoz Inc., Hanover, NJ, USA) and stored at -40°C. CsG metabolites were isolated from the urine using the techniques previously described for the isolation of CsA metabolites in Chapter 1 (192). CsG metabolites were isolated from the urine following diethyl ether extraction by reverse phase HPLC, with eluent fractions corresponding to the peaks of interest collected and pooled.

B. IDENTIFICATION AND CONFIRMATION OF PURITY OF THE CsG METABOLITES

The identity and purity of the isolated metabolite fractions were determined using a similar protocol to that described for CsA metabolites in Chapter 1. FAB/MS, proton-, and in some instances ^{13}C-NMR were used. The lack of availability of large quantities of metabolites prevented analysis of some metabolites by the latter technique.

C. IMMUNOLOGICAL STUDIES

The effect of CsG and its metabolites was investigated using various in vitro immunological systems as previously described for CsA metabolites. Briefly, the inhibition of lymphocyte proliferation by CsG and its metabolites was investigated using a primary and secondary mixed lymphocyte culture as well as a mitogen-stimulated culture system as previously described in Chapter 3. The concentration of CsG and metabolites used to generate dose response curves ranged from 2 to 30,000 μg/L. The 50% inhibitory concentration (IC_{50}) was determined for CsG and each metabolite. The ratio of each metabolite was compared with that of CsG, i.e. IC_{50} of CsG/IC_{50} of metabolite. All experiments were performed in quadruplicate on three separate days.

The concentration of CsG metabolites was determined using HPLC by comparing the area of the metabolite in question to that of an internal standard (CsC), as described in Chapter 1 for CsA metabolites. It was assumed that the CsG and its metabolites exhibited similar molar absorption coefficients. The concentration of all metabolite stock solutions was confirmed by HPLC.

D. CROSS-REACTIVITY STUDIES

Drug-free whole blood obtained from healthy volunteers was spiked with individual CsG metabolites to a final concentration of 100, 500, and 5,000 μg/L. CsG metabolite concentrations were measured as CsG by RIA (Sandimmune RIA, Sandoz Inc., Basel, Switzerland) using either a monoclonal selective or monoclonal non-selective antibody for CsA, as previously described (190,195). Previous studies had demonstrated

that the monoclonal antibodies used in these assays exhibited similar reactivity to both CsA and CsG. Thus these antibodies can be used to measure blood levels of CsG. The percentage cross-reactivity was determined using the following equation: (metabolite recovered as measured as CsG/metabolite concentration added) x 100. Each metabolite was analyzed in duplicate on two separate occasions.

RESULTS

A. FAB/MS AND NMR STUDIES OF CsG

Initially, the FAB/MS and NMR spectra of CsG were compared to CsA. The proton spectrum of CsG was almost identical to that of CsA. The FAB/MS spectrum showed a MH^+ parent ion of 1217 Da (data not shown), as compared to 1203 Da for CsA. This 14 Da difference is due to the substitution of norvaline for α-aminobutyric acid at amino acid 2. The ^{13}C-NMR spectra of CsA and CsG are shown in Figure 22. There are four areas where the spectra differ significantly (these areas are marked by arrows). All of these differences in chemical shifts are a result of the change at amino acid 2. By using the chemical shift data for CsA (97), the chemical shifts for all carbon atoms of CsG were assigned and are listed in Table 10.

B. ISOLATION AND STRUCTURAL IDENTIFICATION OF CsG METABOLITES

A representative chromatogram of the urine profile from subjects receiving CsG and the peaks which were subsequently isolated is shown in Figure 23. The strategy used to assign the structure of each

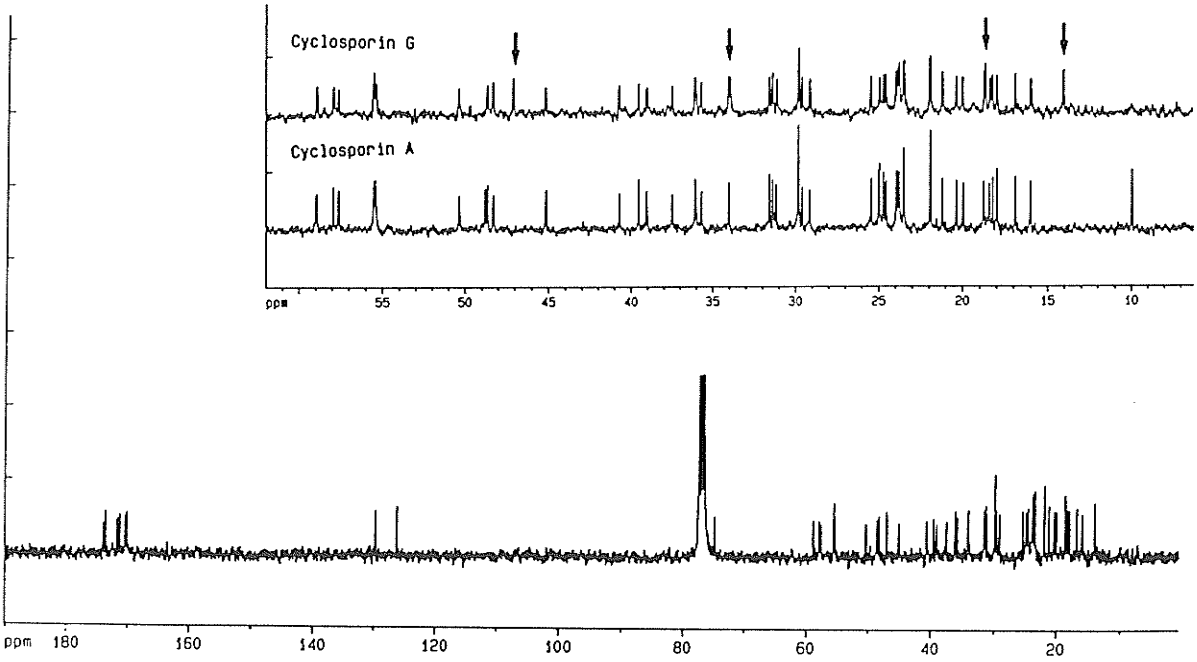

Figure 22. The ^{13}C-NMR spectrum of Cyclosporin G. The insert compares the spectra of CsA and CsG from 10 to 60 ppm. The arrows mark the only four differences between the spectra obtained with CsA and CsG. These differences are attributable to the differences in the carbon atoms of amino acid 2.

Table 10. ^{13}C-NMR chemical shift data obtained for CsG and its metabolites.

Position			CsA[a]	CsG	GM1	GM1c	GM9
AA-1	CH_3N		33.97[b]	34.0	34.1	32.2	34.1
	H-C	(α)	58.75	58.9	57.6	58.5	58.8
	H-C	(β)	74.74	74.6	72.6	82.0	74.8
	H-C	(γ)	35.99	36.0	32.4	36.8	36.0
	H-C	(δ)	35.63	35.8	32.1	41.2	35.7
	CH_3	(γ)	16.76	16.9	17.5	17.5	16.9
	H-C	(ε)	129.68	129.7	132.0	57.0	129.5
	H-C	(ζ)	126.32	126.2	130.0	39.5	126.0
	CH_3	(η)	17.96	18.0	63.5	60.1	18.0
AA-2	H-C	(α)	48.86	47.1	47.0	47.0	47.0
	H-C	(β)	25.06	33.7	33.9	34.7	33.9
	H-C	(γ)	9.93	18.7	18.6	19.2	18.6
	H-C	(δ)	--[c]	14.0	14.1	14.0	14.0
AA-3	CH_3N		39.40	39.4	39.4	38.4	39.5
	H-C	(α)	50.37	50.4	50.1	50.1	50.4
AA-4	CH_3N		31.32	31.3	31.4	31.2	31.2
	H-C	(α)	55.51	55.5	55.6	55.0	55.6
	H-C	(β)	35.99	36.1	36.1	35.8	36.2
	H-C	(γ)	24.90	24.9	24.9	24.9	24.9
	CH_3	(δ)	23.49	23.5	23.7	23.7	23.4
	CH_3	(δ')	21.18	21.2	21.1	21.6	21.1
AA-5	H-C	(α)	55.39	55.5	55.4	54.6	55.7
	H-C	(β)	31.17	31.1	31.3	31.0	30.9
	CH_3	(γ)	19.81	19.9	19.8	19.6	19.9
	CH_3	(δ)	18.48	18.5	18.2	18.6	18.4
AA-6	CH_3N		31.53	31.6	31.6	31.3	31.5
	H-C	(α)	55.31	55.4	55.6	54.5	55.4
	H-C	(β)	37.41	37.5	37.5	36.9	37.5
	H-C	(γ)	25.40	25.4	24.8	25.1	25.6
	CH_3	(δ)	23.87	23.8	24.1	23.9	23.9
	CH_3	(δ')	21.93	21.9	21.8	21.8	22.2
AA-7	H-C	(α)	48.69	48.7	48.4	48.5	48.6
	H-C	(β)	16.07	16.1	15.5	16.2	16.0
AA-8	H-C	(α)	45.20	45.2	44.8	45.2	45.2
	H-C	(β)	18.19	18.2	17.9	17.8	18.0

Table 10. ^{13}C-NMR chemical shift data obtained for CsG and its metabolites. (continued)

Position			CsA	CsG	GM1	GM1c	GM9
AA-9	CH_3N		29.65	29.7	29.7	29.8	29.6
	H-C	(α)	48.30	48.3	48.0	48.4	47.5
	H-C	(β)	39.04	39.1	39.1	38.1	42.5
	H-C	(γ)	24.70	24.7	24.7	24.8	69.6
	CH_3	(δ)	23.74	23.7	23.8	23.7	30.5
	CH_3	(δ')	21.86	21.9	21.3	21.9	30.1
AA-10	CH_3N		29.83	29.8	30.0	30.1	30.0
	H-C	(α)	57.54	57.7	58.5	57.7	57.6
	H-C	(β)	40.73	40.8	40.6	40.8	40.5
	H-C	(γ)	24.55	24.5	24.4	24.6	24.6
	CH_3	(δ)	23.85	23.9	23.8	23.9	24.0
	CH_3	(δ')	23.38	23.4	23.5	23.3	23.4
AA-11	CH_3N		29.81	29.8	29.8	30.0	29.9
	H-C	(α)	57.93	57.9	58.3	58.2	57.8
	H-C	(β)	29.05	29.1	29.1	29.2	29.0
	CH_3	(γ)	18.75	18.6	18.6	18.6	18.6
	CH_3	(δ)	20.26	20.3	20.4	20.1	20.1

[a] Literature values reported by Kessler et al (97).

[b] Value represents the chemical shift (δ) in ppm.

[c] No carbon atom found for CsA.

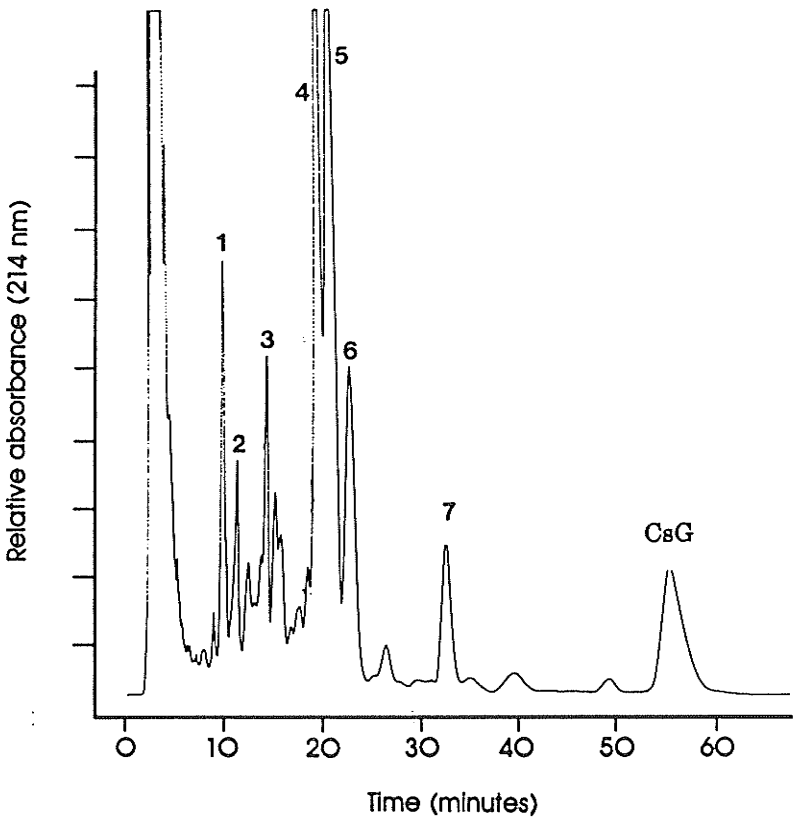

Figure 23. HPLC profile of CsG metabolites isolated from the urine of normal subjects receiving the drug. Peaks 1 to 7 represent the individual metabolites isolated for subsequent characterization. CsG elutes at approximately 55 minutes as indicated. No other peaks eluted after this time.

metabolite was similar to that described for CsA metabolites (Chapter 1) and is presented in detail below. Table 11 summarizes the structural characteristics for each individual metabolite. In addition, the corresponding CsA metabolite similarly modified is designated. The purity of each metabolite fraction was >97% using the criteria described for CsA metabolites in Chapter 1.

Peak 1: The protonated molecular ion of this metabolite was observed using FAB/MS as a m/z of 1249 (MH^+), corresponding to a 32 Da increase of molecular mass as compared to the protonated molecular ion of the parent drug, CsG (m/z 1217 MH^+). A fragment of m/z of 1120 Da was observed resulting from the loss of a 129 Da fragment from the protonated molecular ion, as compared with a loss of 113 Da for CsG, suggesting that an additional oxygen was present at amino acid 1 (AA-1). Proton-NMR analysis confirmed this modification at AA-1, as demonstrated by the disappearance of the η-CH_3 peak usually observed for this amino acid. The latter procedure also indicated that none of the N-CH_3 groups were demethylated, as evidenced by the presence of seven N-CH_3 singlets and four N-H doublets. This metabolite was identified as being dihydroxylated with hydroxylation occurring at AA-1, and probably AA-9. Hydroxylation at this latter site would have been confirmed if significant metabolite was available for ^{13}C-NMR analysis. It should be noted that the proton-NMR spectrum for this metabolite was remarkably similar to that for CsA metabolite M-8, which is hydroxylated at AA-1 and AA-9. This metabolite was designated as GM19 using the nomenclature recently proposed for CsA

Table 11. Characteristics of isolated CsG metabolites.

Metabolite peak	Metabolite[a]	Molecular weight$_{+}$(Da) (MH^{+})	Modification	CsA metabolite equivalent
1	GM19	1249	Hydroxylated AA-1[b], AA-9	M-8
2	GM1c9	1249	Hydroxylated AA-1, AA-9 Cyclization AA-1 (?)	M-26
3	GM4N9	1219	Hydroxylated AA-9 Demethylated AA-4	M-13
4	GM1	1233	Hydroxylated AA-1	M-17
5	GM9	1233	Hydroxylated AA-9	M-1
6	GM1c	1233	Hydroxylated AA-1 Cyclization AA-1	M-18
7	GM4N	1203	Demethylated AA-4	M-21

[a] Metabolite identified using the nomenclature recently proposed for cyclosporine analogues (139).

[b] AA-1 = Amino Acid 1.

analogues (139).

Peak 2: The FAB/MS of metabolite peak 2 indicated a molecular ion species of m/z 1249 (MH^+) corresponding to an increase of 32 Da over CsG, which is consistent with the addition of two oxygen atoms. A fragment of m/z 1120 Da was observed resulting from the loss of a 129 Da fragment from the protonated molecular ion, as compared with a loss of 113 Da for CsG, suggesting that an additional oxygen was present at AA-1. The proton-NMR confirmed the modification of AA-1. Due to lack of sufficient material, ^{13}C-NMR analysis of this metabolite was not possible. Its proton-NMR spectrum was almost identical to that of CsA metabolite M-26. This metabolite was identified as being hydroxylated at AA-1 and probably AA-9, and was tentatively identified as GM1c9 according to the proposed nomenclature (139).

Peak 3: The protonated molecular ion was at m/z 1219 (MH^+) which is a 2 Da increase over CsG. This is considered to be due to the addition of one oxygen (+16 Da) and the loss of one methyl group (-14 Da). The presence of a fragment of m/z 1106 (-113 Da) indicated that there was no modification of the side chain of AA-1. The above findings were confirmed by proton-NMR. There was no loss of the η-CH_3 peak of this amino acid, verifying that no modification had occurred at this amino acid. In addition, a loss of one of the seven N-CH_3 singlets corresponding to AA-4, with the appearance of a fifth N-H doublet indicated that this metabolite was demethylated at AA-4.

There was not sufficient metabolite for analysis by ^{13}C-NMR. This metabolite was identified as being demethylated at AA-4 and hydroxylated at AA-9 (not confirmed), and designated as GM4N9 according to the proposed nomenclature (139).

Peak 4: The protonated molecular ion was at m/z 1233 (MH^+) Da, a 16 Da increase over CsG, attributable to the addition of one oxygen atom. The FAB/MS spectrum showed a fragment of 1104 Da (-129 Da) suggesting that hydroxylation had occurred at AA-1. Proton-NMR confirmed the modification at this site. This was evidenced by the loss of the η-CH_3 peak at 1.62 ppm, normally found in CsG. The FAB/MS and proton-NMR spectra of this metabolite are shown in Figure 24. Analysis of the ^{13}C-NMR spectrum of this metabolite confirmed the modification of the η-CH_3 carbon of AA-1. This was evidenced by the shift in the η-CH_3 peak of AA-1 from 18.0 ppm to 63.5 ppm (see Table 10). A comparison of the ^{13}C-NMR spectra of CsG and this metabolite is shown in Figure 25. This metabolite was identified as being hydroxylated at AA-1 and designated as GM1 according to the proposed nomenclature (139). The ^{13}C-NMR spectrum of GM1 is remarkably similar to that of CsA metabolite, M-17. A comparison of the spectra is shown in Figure 26. The only differences between the two spectra are those attributable to AA-2 (the differences between parent CsA and CsG). This can better be seen by comparing the chemical shifts for the two metabolites (Table 4 for M-17; Table 10 for GM1).

Peak 5: The protonated molecular ion was at m/z 1233 (MH^+),

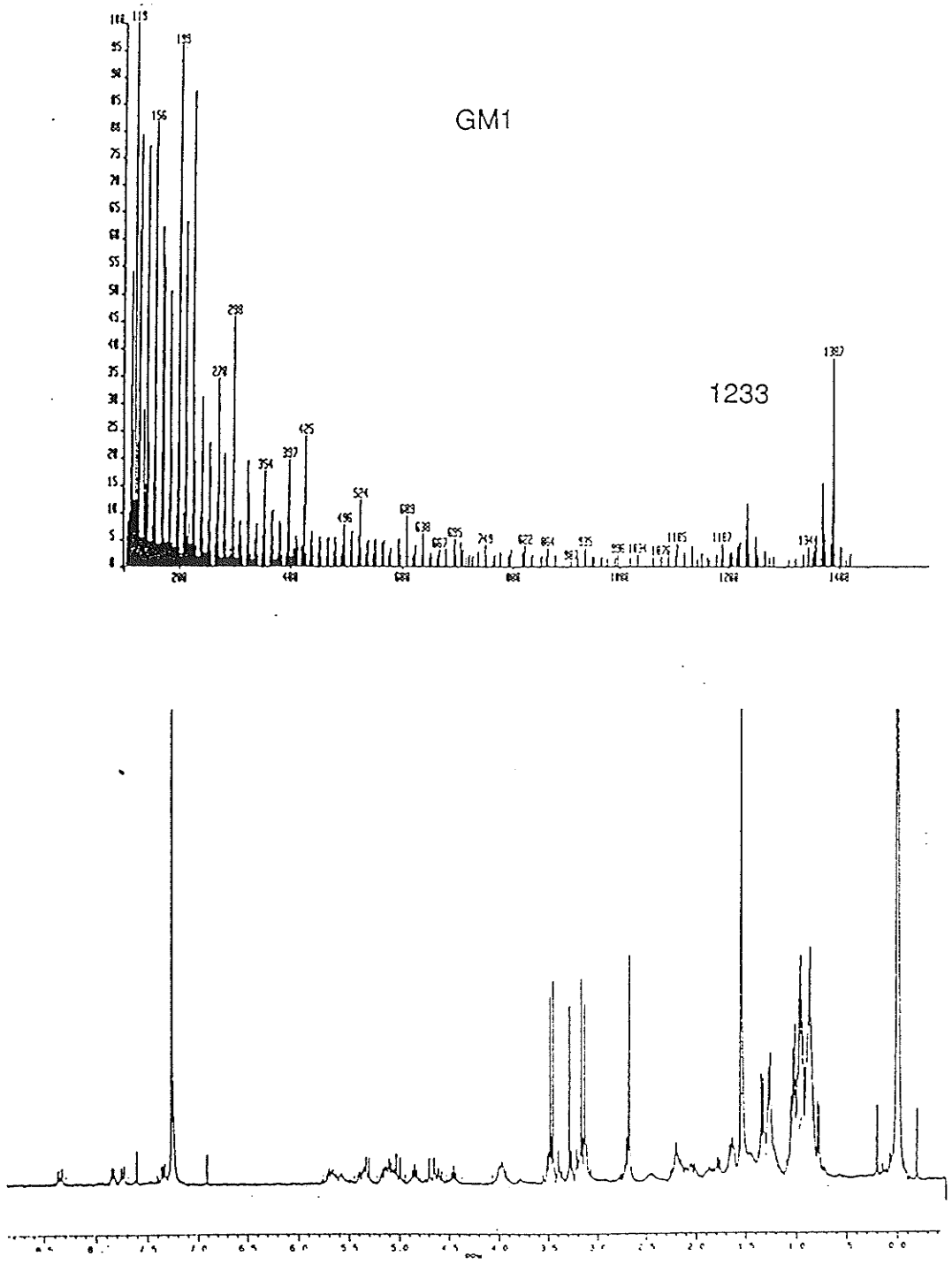

Figure 24. FAB/MS and proton-NMR spectra of CsG metabolite GM1. The FAB/MS spectrum (Top) produced a parent ion of MH^+ 1233 Da. The peak at 1387 Da is a result of this metabolite and the matrix (dithiothreitol) producing a peak 154 Da larger.

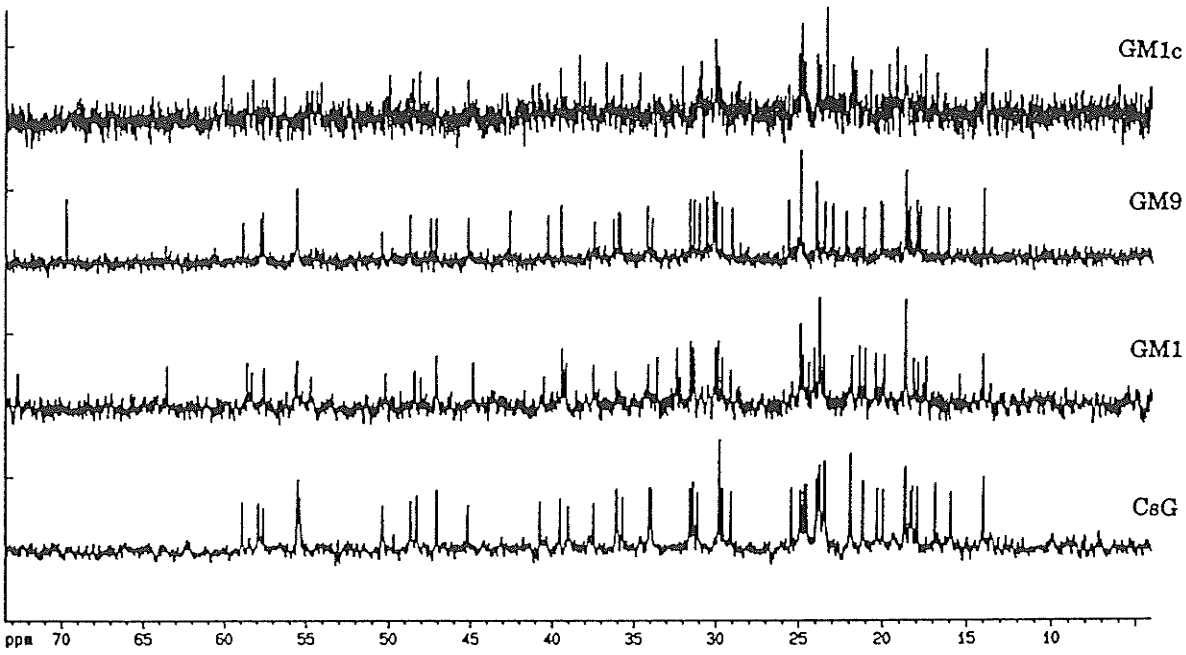

Figure 25. Comparison of ^{13}C-NMR spectra of CsG and metabolites GM1c, GM9, and GM1. The spectra from 0 to 75 ppm are displayed.

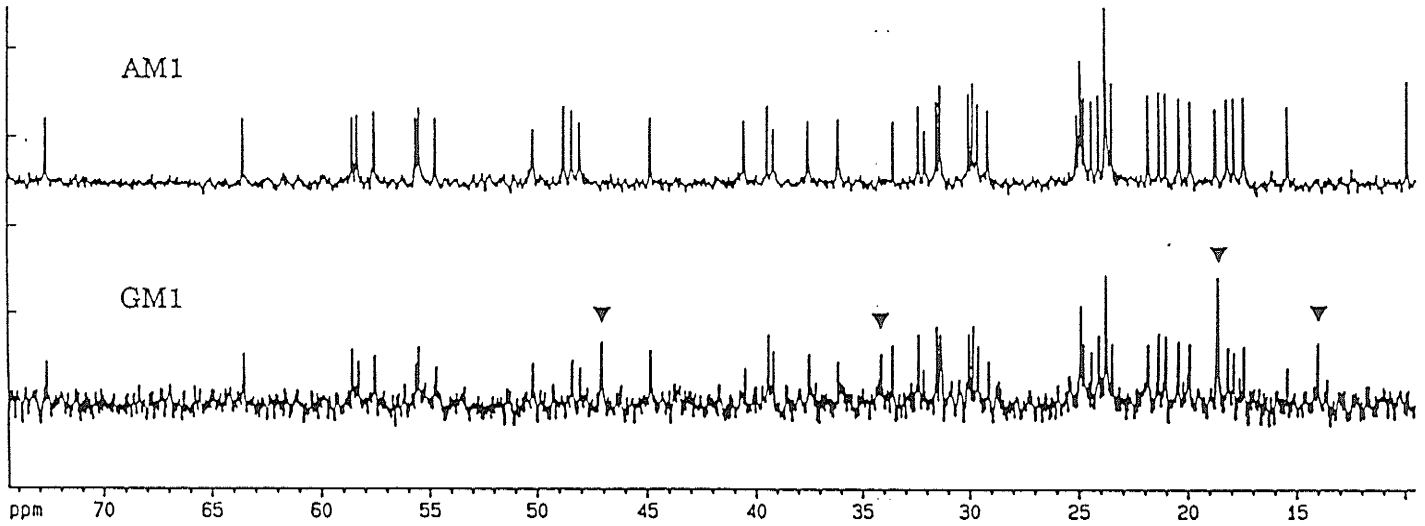

Figure 26. Comparison of ^{13}C-NMR spectra of the CsA metabolite AM1 and the CsG metabolite GM1. The spectra from 10 to 75 ppm are displayed. The arrows indicate the major differences between the two metabolites.

which was a 16 Da increase over CsG. The presence of a 1120 fragment (-113 Da) indicated that no modification had occurred at AA-1. This was confirmed by proton-NMR analysis, where no loss of the η-CH_3 peak of AA-1 was observed. ^{13}C-NMR (see Figure 25 and Table 10) confirmed that hydroxylation had occurred at the γ-carbon of AA-9. This was evidenced by the chemical shift of this carbon from 24.7 to 69.6 ppm. No other major differences in the ^{13}C-NMR spectra of this metabolite were observed, other than minor shifts occurring at AA-9. This metabolite was identified as being hydroxylated at AA-9 and designated as GM9 according to the proposed nomenclature (139).

Peak 6: The protonated molecular ion was a m/z 1233 (MH^+), a 16 Da increase over CsG. The presence of a fragment of 1104 (-129 Da) indicated that modification had occurred at AA-1. This finding was confirmed by proton-NMR (loss of η-CH_3 peak of AA-1). ^{13}C-NMR analysis (see Figure 25 and Table 10) indicated significant modification at AA-1. The loss of the vinylic carbons of this amino acid suggests cyclization and subsequent rearrangement of AA-1. Furthermore, shift of the η-CH_3 peak from 18.0 to 60.1 ppm indicated hydroxylation had occurred at AA-1. This metabolite was therefore identified as being hydroxylated at AA-1 with cyclization at this amino acid also occurring and was designated as GM1c according to the proposed nomenclature (139). The ^{13}C-NMR spectra of this metabolite was almost identical to that of CsA metabolite M-18 [compare ^{13}C-NMR chemical shifts for M-18 (Table 4) and AM1c (Table 10)], thus further confirming its identity.

Peak 7: The protonated molecular ion was at m/z 1203 (MH^+), a 14 Da decrease from CsG, which was ascribed to the loss of one methyl group (-14 Da). A fragment of 1090 (-113 Da) indicated that no modification had occurred at AA-1. The proton-NMR indicated a loss of one of the N-CH_3 singlets corresponding to AA-4, with an additional N-H doublet being observed. This metabolite was designated as GM4N according to the proposed nomenclature (139).

C. IMMUNOSUPPRESSIVE ACTIVITY OF CsG METABOLITES

The immunosuppressive activity of CsG, its metabolites, as well as CsA, were tested on three separate occasions using primary (1°) and secondary (2°) mixed lymphocyte cultures as well as a phytohemagglutinin-stimulated culture. Table 12 shows the concentration of the metabolites that result in 50% inhibition of [^{3}H]-thymidine uptake (IC_{50}) in all three test systems. The potency ratio of the IC_{50} of metabolites to that of CsG are also shown. The IC_{50} of CsG, its metabolites, as well as CsA, varies from system to system. In all cases significantly more drug, parent or metabolite, was required to inhibit the phytohemagglutinin mitogen-stimulated system as compared to the alloantigen-stimulated systems. In all three systems CsA was found to be 2 to 3 fold more immunosuppressive than CsG. The singly modified metabolites GM1, GM9, and GM4N were the most immunosuppressive metabolites examined. However, regardless of assay, they were generally <10% as effective as CsG in inhibiting proliferation. All other metabolites tested were less than 1 to 2% as immunosuppressive as CsG.

Table 12. Immunosuppressive activity of CsG metabolites.

Metabolite peak	Metabolite	IC_{50}, µg/L[a] 1° MLC		2° MLC		Phytohemagglutinin	
1	GM19	6550 ± 250	(<0.01)[b]	>18,000	(<0.01)	>30,000	(<0.01)
2	GM1c9	>18,000	(<0.01)	>18,000	(<0.01)	>30,000	(<0.01)
3	GM4N9	3550 ± 120	(0.01)	5630 ± 900	(<0.01)	24,100 ± 600	(<0.01)
4	GM1	329 ± 99	(0.10)	620 ± 60	(0.06)	2440 ± 465	(0.05)
5	GM9	403 ± 78	(0.08)	540 ± 30	(0.07)	2350 ± 630	(0.05)
6	GM1c	3300 ± 220	(0.01)	11,300 ± 940	(<0.01)	24,100 ± 600	(<0.01)
7	GM4N	425 ± 103	(0.08)	760 ± 50	(0.05)	3020 ± 830	(0.04)
CsG		33 ± 6	(1.00)	40 ± 4	(1.0)	123 ± 31	(1.00)
CsA		15 ± 3	(2.2)	13 ± 2	(3.07)	42 ± 6	(2.92)

[a] Results are expressed as mean (and SD) of three measurements done on separate days.

[b] Potency of metabolite relative to CsG (CsG = 1.0) is listed in parentheses.

D. CROSS-REACTIVITY OF CsG METABOLITES

Table 13 summarizes the cross-reactivity data obtained for the isolated CsG metabolites with the Sandoz selective and non-selective monoclonal antibodies. Previous studies in our laboratory demonstrated that CsG cross-reacted 100% with both of these antibodies (data not shown). The majority of CsG metabolites examined exhibited little cross-reactivity with the selective antibody; GM9 exhibited 10% cross-reactivity, whereas GM1, GM1c, and GM4N exhibited 2 to 3% cross-reactivity. The remaining metabolites examined had <1% cross-reactivity. The metabolites exhibited cross-reactivity to varying degrees with the poly-selective antibody. GM1c was the most cross-reactive metabolite examined, with values of 120%. The other metabolites examined ranged from 70% for GM1 and GM9 to 6% for GM4N9.

DISCUSSION

In view of the severe nephrotoxicity associated with CsA therapy, there has been much interest in the search for CsA analogues which are less nephrotoxic but still retain significant immunosuppressive properties. One analogue which may show promise is cyclosporin G (CsG). CsG is a naturally occurring analogue of cyclosporin A (CsA) in which norvaline has replaced α-aminobutyric acid at the amino acid 2 position of the molecule (94). Preliminary work in animals suggests that CsG is as immunosuppressive but not as nephrotoxic as CsA, although conflicting reports do exist (179-185). Clinical trials on the use of CsG in prevention of allograft rejection in renal transplantation as well as in the treatment of autoimmune diseases

Table 13. Cross-reactivity[a] of CsG metabolites.

Metabolite	Assay: Sandimmune selective	Assay: Sandimmune non-selective
GM19	<1%[b]	26.8%
GM4N9	<1%	6.0%
GM1	2.6%	71.5%
GM9	9.8%	68.4%
GM1c	2.1%	120.7%
GM4N	3.4%	18.7%

[a] Cross-reactivity was determined by (metabolite recovered as measured as CsG/metabolite added) x 100.

[b] Value represents the mean of duplicate experiments performed on two separate days.

such as uveitis are presently underway.

To date, there have been no published reports on the structure and immunosuppressive properties of CsG metabolites. In future clinical trials it will be important to know whether CsG metabolites should be monitored in patients receiving the drug to allow appropriate dosage adjustments to be made to maximize immunosuppression while minimizing toxicity. As with CsA, the role of CsG metabolites in overall immunosuppression and toxicity must be elucidated prior to answering these questions. This study was designed to identify the structure as well as to assess the in vitro immunosuppressive effects of the various CsG metabolites that appear in the urine from subjects receiving the drug.

Seven major metabolites of CsG were isolated from the urine of normal subjects receiving a single injection of the drug. In addition to the metabolites isolated, there appear to be a number of additional peaks on the HPLC chromatogram which may also be CsG metabolites. The structure and purity of the isolated metabolites was assessed by FAB/MS, proton-NMR, and in most cases ^{13}C-NMR. All the metabolite fractions were assessed to be free of contamination (>97% pure). The use of these techniques in the structural determination of CsA metabolite structure and in the assessment of purity has previously been discussed. The modifications observed for CsG metabolites primarily occurred on amino acid 1 and 9 (hydroxylation) and amino acid 4 (demethylation). In addition, metabolites in which amino acid 1 was cyclized were also identified (i.e. GM1c, GM1c9). The metabolites were named according to a nomenclature system recently

adopted for CsA metabolites (139). The use of a standardized nomenclature will provide a systematic approach for naming of additional metabolites as they are identified. The structures reported for CsG metabolites identified so far are consistent to what has been identified for CsA metabolites as previously discussed in Chapter 1. This is not entirely surprising due to the similarities in structure and conformation between the two molecules.

Due to the structural similarities of CsG metabolites with those of CsA, it appears that CsG is metabolized by the same cytochrome P-450 isoenzymes required for CsA metabolism (105,116-120). Two isoenzymes have been shown to be responsible; first, the P-450 IIIa, or the P-450 NF, which is responsible for the metabolism of many hydrophobic compounds including nifedipine, cortisol, erythromycin, and quinidine (117-119). A second cytochrome, P-450 hpCN3, has also been found to be involved in CsA metabolism (120). Further, the biotransformation of CsG is likely to be similar to CsA. For example, GM1, GM9, and GM4N are likely to be primary metabolites, with the other metabolites arising due to further metabolism of these three metabolites. However, direct proof of these enzymes in CsG metabolism is required to further address this question.

To date the immunosuppressive properties of CsG metabolites and their role in overall immunosuppression in vivo are not known. The immunosuppressive activities of CsA, CsG, and its metabolites were tested simultaneously in three separate in vitro assay systems. The use of three test systems, the same metabolite stocks between experiments, and the expression of results as a potency ratio of IC_{50}

values have allowed for a valid comparison of the immunosuppressive activities of CsG metabolites. The immunosuppressive activity of CsG was found to be 2 to 3 fold less than that of CsA. This difference in activity has previously been reported by other investigators (178). The immunosuppressive potency of CsG metabolites was less than that of the parent drug. It was found that the immunosuppressive activities of the CsG metabolites examined varied between the assay systems used. However all metabolites examined exhibited activity <10% of that of CsG. Metabolites with single modifications, GM1, GM9 (hydroxylation), or GM4N (demethylation), possess the most immunosuppressive activity. Metabolites with two or more modifications possess substantially less. The immunosuppressive potencies with respect to parent drug for the CsG metabolites is consistent to what has been reported for CsA metabolites (93,94,121,131,144-150). Despite the fact that CsG is 3 fold less immunosuppressive than CsA, the relative immunosuppressive activity of CsG metabolites to parent drug is the same as CsA metabolites to parent drug.

The monitoring of whole blood levels of CsG can be performed using either HPLC or RIA. The Sandimmune RIA for CsA demonstrates 100% cross-reactivity with CsG. The monoclonal selective antibody demonstrates minimal cross-reactivity with CsG metabolites, thus this assay can be used for specific measurements of CsG. The monoclonal non-selective antibody demonstrates significant cross-reactivity, thus it will measure CsG plus a variety of metabolite concentrations to different degrees. At this time it is not known whether measurement of parent drug alone or parent drug plus metabolites provides a better

indication of clinical condition.

To date, nothing is known about the role of CsG metabolites in toxicity. This question is of particular importance in the overall use of CsG in organ transplantation. In cases such as liver transplantation, high concentrations of CsA metabolites may have a significant role in hepatotoxicity (172). Given the similar biotransformation of CsG as compared to CsA, the monitoring of CsG metabolites in liver transplantation may be warranted. In this situation altered hepatic function can substantially alter CsA metabolism, and presumably CsG metabolism, giving rise to a highly variable metabolite to parent drug ratio. Experiments are required to assess the role of CsG metabolites in the pathogenesis and time course of CsG-induced toxicity.

A number of clinical studies have been proposed or are presently underway which will test the efficacy of CsG in solid organ transplantation as well as in the treatment of autoimmune diseases. The fact that the above findings suggest that CsG metabolites are $<10\%$ as active as CsG indicates that monitoring of parent drug rather than parent drug plus metabolites may be sufficient as a guideline for dosage adjustment.

VIII. CONCLUSIONS

Historically, the potential importance of CsA metabolites in the overall management of patients receiving the drug has not been thoroughly elucidated, due in part to the lack of sufficient quantities of metabolites required for both in vitro and in vivo studies. It is still unclear as to whether CsA alone or CsA plus metabolites should be monitored in patients receiving the drug to allow better dosage adjustments to be made to minimize the toxic effects of the drug, while maximizing immunosuppression. The evidence presented in this thesis allows several conclusions to be drawn regarding the potential role of CsA metabolites in the clinical management of such patients.

In order to investigate the in vitro effects of CsA metabolites, procurement of sufficient metabolites of known structure and purity is required, something which has not been available in the past. The use of such material is required if meaningful results are expected to be obtained. In this thesis, the use of techniques such as FAB/MS and both proton- and ^{13}C-NMR have allowed for the positive identification and assessment of purity of isolated CsA metabolites.

The use of well characterized pure metabolites, along with three different in vitro tests to assess immunosuppression, allowed for a thorough investigation into the immunosuppressive activity of several major CsA metabolites. The expression of the results as a potency ratio with respect to parent CsA has allowed for a valid comparison of the potencies of the metabolites to CsA. These in vitro experiments

indicate that CsA metabolites are considerably less immunosuppressive than the parent drug. Only the primary metabolites of CsA, M-1, M-17, and M-21 were demonstrated to possess any immunosuppressive activity, albeit they were only 10 to 15% as active as the parent drug regardless of which system was used to assess activity. The data are still inconclusive on the role that CsA metabolites may play with respect to immunosuppression in vivo. In vivo studies in which purified metabolites alone or in combination are administered, with allograft rejection being monitored, are required before significant conclusions can be drawn with regards to the role of CsA metabolites in overall immunosuppression. The procurement of sufficent quantities of metabolites has prevented such studies.

The investigation into the pharmacokinetics of the major CsA metabolite, M-17, allowed valuable information to be obtained with regards to its biotransformation. The demonstration that M-17 undergoes further metabolism to several other CsA metabolites confirms the proposed biotransformation pathways of CsA. In addition, the clearance and distribution of M-17 is similar to that of the parent compound, despite the fact that its tissue concentrations are higher than those of the parent drug. This raises the possibility that CsA undergoes metabolism in tissues such as the kidney. The implication of this in CsA nephrotoxicity poses important questions with regards to its clinical relevance.

Historically, in vitro assay systems have been used to investigate the toxic effects of CsA; however, very few studies have investigated the toxic effects of CsA metabolites. In this thesis the

toxicity of several major CsA metabolites was investigated using a variety of cytotoxic and functional parameters in two cell lines, the $LLC\text{-}PK_1$ cell line and a primary rabbit mesangial cell line. In general, CsA metabolites were not toxic with regards to the metabolic parameters examined in these in vitro systems. Two metabolites exhibited moderate cytotoxic properties, M-1 and M-21, although not to the same degree as CsA. To further investigate the toxicity of CsA metabolites, the effect of CsA and metabolites was studied on the release of two potent vasoactive substances from mesangial cells. CsA and metabolites were equally effective in inhibiting the release of prostacyclin from the mesangial cells in a concentration-dependent manner. In addition, CsA did not affect the release of endothelin from these cells, however M-8 and M-26 resulted in increased production. These preliminary findings suggest that some CsA metabolites have the potential to affect the hemodynamic control of the vasculature, suggesting a potential role in the development of CsA toxicity.

Because of the nephrotoxic effects of CsA, there has been much interest in the search for CsA analogues which are less toxic but still retain their immunosuppressive activity. The finding that metabolite toxicity does not positively correlate with immunosuppression suggests that the potential of finding such an analogue may exist. Besides metabolites of CsA itself, several other CsA analogues have been developed. One such analogue is CsG. Preliminary studies have suggested CsG may be less nephrotoxic than CsA, will still retaining significant immunosuppressive activity. In

view of this, studies were undertaken to investigate its metabolites in a manner similar to CsA. CsG appears to be metabolized by the same P-450 isoenzymes required for CsA metabolites, since the metabolite profile is similar to that of CsA. In addition, the in vitro immunosuppressive activity of CsG metabolites was substantially less than that of parent CsG, paralleling that of CsA and its metabolites.

In conclusion, the results of this thesis provide no support for the routine monitoring of CsA metabolites at this time. In addition, in subsequent studies with CsG, the monitoring of CsG metabolites may not be warranted. Thus, monitoring of parent drug, whether it be CsA or CsG, should provide clinicians with a guideline on which to dose the drug to maintain maximal efficacy of the drug. Although some clinical studies have implied a role of CsA metabolites in CsA toxicity, to date no concrete evidence has been provided. The finding that changes in metabolite concentrations are associated with toxicity does not necessarily mean a cause-effect relationship. However, pending unequivocal documentation that metabolites are not active, the overall role of CsA metabolites in CsA therapy will remain uncertain.

The long-term goals of transplant physicians is to decrease the incidence and severity of CsA-induced nephrotoxicity. This can be accomplished through a thorough understanding of the etiology of CsA nephrotoxicity. In addition, the discovery of a CsA analogue which is immunosuppressive but less toxic than CsA will have a significant impact on the quality of care of transplant patients. Recent interest has focussed on the development of protocols in which CsA is administered at reduced doses in combination with other potentially

clinically relevant drugs. In this regard several new immunosuppressive agents are being examined, including Rapamycin and FK-506. Thus the foundations for the development of new clinical protocols will ultimately lead to improved patient care.

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