Immunosuppressive Activity of Cyclosporine Metabolites Compared and Characterized by Mass Spectroscopy and Nuclear Magnetic Resonance

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Eight cyclosporine (CsA) metabolites were isolated from the urine of renal-transplant patients by high-pressure liquid chromatography. Structure and purity of the metabolites were assessed by fast atomic bombardment/mass spectroscopy, by proton nuclear magnetic resonance (NMR), and, when the quantity of metabolites permitted, by 13C-NMR. The immunosuppressive activities (I) of the metabolites were tested in three separate in vitro systems: primary and secondary mixed lymphocyte reactions as well as by a mitogen-stimulated system. The I, as measured by comparing the concentration of each metabolite required for 50% inhibition of incorporation of [3H]thymidine, varied among the assay systems, as did the ranking of I among the test systems. In general, the I of most metabolites in all assay systems were <10% of that for CsA. Metabolites with some modifications exhibited the greatest I; e.g., that of M-17 was ~16% of that of CsA (potency ratio 0.16) in a secondary mixed lymphocyte reaction. The significance of these findings in relation to therapeutic monitoring of CsA is discussed.

Additional Keyphrases: kidney-transplant recipients • urine • lymphocyte cell cultures • radioassay • monitoring therapy • method-related differences

Cyclosporine (CsA) is extensively metabolized by the hepatic P-450 {EC 1.14.15.6}; cholesterol monooxygenase (side-chain-cleaving) system (1, 2). Approximately 24 metabolites resulting from the biotransformation of CsA have been isolated from urine and bile and chemically characterized (1–6). The concentration of some metabolites can exceed that of unchanged ("parent") CsA both in blood and, in particular, tissues of transplant recipients (7, 8). Yet the role of the metabolites in overall immunosuppression and toxicity remains uncertain. This information is clinically important, for eventually it will become clear whether one or more metabolites should be monitored along with parent CsA as a guide for dosage adjustments to minimize toxicity while maximizing immunosuppression.

The measured amount of immunosuppressive activity of various CsA metabolites has varied from study to study (1, 3, 4, 9–16), but has always been less for each metabolite than for the parent drug. Reported immunosuppressive activity of the major CsA metabolite, M-17, has ranged from 10% to 80% of that of CsA (1, 3, 4, 9–16). This disagreement may be attributed to several reasons, including the uncertain purity of the metabolites. In some instances, only high-pressure liquid chromatography (HPLC) has been used to assess purity (1, 3, 4, 9–16). Moreover, different in vitro assays have been used to assess immunosuppressive activity (1, 3, 4, 9–16). However, CsA metabolites reportedly (10) have a relatively greater inhibitory effect on alloreactive T cells, including those found in cellular infiltrates of allografts, than on the primary activation of T cells.

We report here a comparison of the in vitro immunosuppressive properties of a large series of CsA metabolites isolated from the urine of renal-transplant recipients. The structure and purity of the metabolites were confirmed by fast atomic bombardment/mass spectroscopy (FAB/MS) as well as 1H-NMR and, in some cases, depending on availability of metabolites, 13C-NMR. We also performed several in vitro assays with human peripheral-blood mononuclear cells (PBMC) to address the problem of methodological differences previously encountered in assessment of immunosuppressive activity.

Materials and Methods

Isolation of Cyclosporine Metabolites

We isolated CsA metabolites from the urine of renal-transplant recipients receiving CsA, by a procedure previously described (17), modified as follows. To a 40- to 50-mL aliquot of urine (pH adjusted to 10.0 with 2 mol/L NaOH) add 50 to 60 mL of "HPLC-grade" diethyl ether, shake the mixture for 10 min, and remove the urine layer. Using the same ether layer, extract two more portions of the urine specimen. Then add 50 mL of 0.2 mol/L HCl to the ether layer and shake the mixture for 10 min. Remove and evaporate the ether layer and reconstitute the residue in 500 µL of mobile phase (acetonitrile/methanol/water; 40/20/40 by vol). After adding 500 µL of hexane to this, vortex-mix the sample for 30 s, then centrifuge (2000 x g, 5 min). Discard the hexane (upper) layer and collect the remaining solvent layer for subsequent separation by HPLC.

For our isocratic chromatographic separation of CsA metabolites we used a Model 5000 chromatograph (Varian Inc., Walnut Creek, CA), with a 25 × 0.46 cm column of 5-μm Spherosorb C-8 particles (Chromatography Sciences Inc., Rexdale, Ontario, Canada)—preceded by two precolumns, one of pellicular silica and the other of RP-8 (Upchurch Scientific, Oak Harbor, WA)—and the mobile phase described above. In some instances, we used a tandem C-8 column and decreased the concentration of acetonitrile in the mobile phase to improve chromatographic resolution. The flow rate of the solvent was 1.0 mL/min; the column temperature was maintained at 70 °C. Eluent fractions corresponding to the peaks were collected manually, and the mobile phase was evaporated under low heat. Corresponding fractions from multiple injections were pooled for subsequent experiments.

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4 Nonstandard abbreviations: CsA, cyclosporine; FAB/MS, fast atomic bombardment/mass spectroscopy; NMR, nuclear magnetic resonance; PMBC, peripheral-blood mononuclear cells; MLC, mixed lymphocyte culture; and IC50, 50% inhibitory concentration.

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Identification and Confirmation of Purity of the Metabolites

Mass spectroscopy. To determine the molecular mass of the metabolites, we used a Model 7070E-HF mass spectrometer (VG MassLab, Altrincham, U.K.) equipped with a fast atomic bombardment source, a postacceleration detector, and a Model 2506 data system. The matrix used was dithiothreitol/dithioerythritol (5:1 by vol) (3). About 5 to 10 \( \mu \)g of metabolite was required for each analysis. Highly pure (99.99%) "research-grade" xenon was used as the bombardment gas, and the resulting positive ions were extracted into the mass analyzer. A mass range of 100 to 1500 Da was scanned.

Nuclear magnetic resonance (NMR). We analyzed the proton and \(^{13}\)C-NMR spectra of the CsA metabolites at 300 MHz in CDCl\(_3\), using a Model WH90/AAM300 NMR (Bruker Instruments Inc., Silverstiegen, F.R.G.) and tetramethylsilane as the internal standard (3).

Immunological Studies

To study inhibition of lymphocyte proliferation by CsA and its metabolites, we used PBMC isolated from heparinized blood by Ficoll–Hypaque density-gradient centrifugation (18). The blood donors were ostensibly healthy hospital personnel. The immunosuppressive activity of CsA and metabolites was examined in three ways, as previously described (19):

Mitogen stimulation. PBMC (1 \( \times \) 10\(^6\)mL\(^{-1}\)) were cultured (100 \( \mu \)L per well) in triplicate for 48 h at 37 °C in a CO\(_2\)-enriched (5% CO\(_2\)) atmosphere in 96-well microtiter plates with phytohemagglutinin, 20 \( \mu \)g/mL, in RPMI-1640 medium (GIBCO Labs., Grand Island, NY 14072) containing 100 mL of AB (blood group) human serum per liter and antibiotics (complete medium), with or without CsA or metabolite. Cultures were then pulsed with \(^{3}H\)thymidine (Amersham Inc., Oakville, Ontario), 0.3 \( \mu \)Ci per well. Eighteen hours later we harvested the cultures and determined their radioactivity by liquid scintillation counting.

Primary mixed-lymphocyte culture (1st MLC). Responder PBMC will proliferate when cultured with PBMC of another HLA phenotype (stimulator). We cultured 100 \( \mu \)L of responder PBMC (1 \( \times \) 10\(^5\)mL\(^{-1}\)) with 100 \( \mu \)L of a pool (10 donors) of irradiated (25 Gy, or 2500 rads) stimulator PBMC in complete medium. The latter were irradiated to prevent their proliferation. Assays were run in quadruplicate in 96-well round-bottom microtiter trays for six days with or without (control) CsA or metabolite, with an 18-h terminal pulse of \(^{3}H\)thymidine (0.3 \( \mu \)Ci per well).

Secondary mixed-lymphocyte culture (2nd MLC). We cultured 10 \( \times \) 10\(^5\)mL responder PBMC with 10 \( \times \) 10\(^5\)mL irradiated (2500 rad) stimulator PBMC (pool of 10 donors) in a total volume of 10 mL of complete medium for six days. The primed cells were then washed and recultured in triplicate (5 \( \times \) 10\(^4\)\( \pm \)100 \( \mu \)L) with the same pool of irradiated stimulator cells (1 \( \times \) 10\(^5\)\( \pm \)100 \( \mu \)L), with or without CsA or metabolite, in 96-well round-bottomed microtiter trays. The cells were incubated for a further 72 h with a 4- to 6-h terminal pulse of \(^{3}H\)thymidine.

The CsA and metabolite preparations used in the experiments above were dissolved in 10 \( \mu \)L of 950 mL/L ("95%") ethanol containing 200 mL of Tween surfactant 80 per liter and diluted with RPMI-1640 to yield the desired concentrations of drug. For a control we prepared RPMI-1640 medium (without drug) containing 0.50 mL of ethanol and 1 \( \mu \)L of Tween 80 per liter. The same stock concentrations for CsA and metabolites were used for all experiments. In preliminary experiments we found that the diluent alone at the highest concentration (equal to the most concentrated CsA sample) had no immunosuppressive effect on the proliferation of the PBMC; therefore, we used it as the control in each experiment. The concentrations of CsA and metabolites used to generate dose–response curves ranged from 2.0 to 20 000 \( \mu \)g/L (CsA and metabolite concentrations confirmed by HPLC). We determined the 50% inhibitory concentration (IC\(_{50}\)) for CsA and each metabolite, then compared the potency ratio of each metabolite with that of CsA, i.e., IC\(_{50}\) of CsA/IC\(_{50}\) of metabolite.

Results

Metabolite Purification

Figure 1 depicts a representative chromatogram of the separated CsA metabolites that were in the ether extract of urine of transplant recipients. Eight of the peaks (A–H) were purified to >99% purity as determined by FAB/MS and NMR. The identities of the peaks listed were established by the following procedures:

Peak A. The protonated molecular ion of this metabolite peak was observed in the FAB/MS of m/z 1235 (MH\(^+\)), corresponding to a 32-Da increase of 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 prototomed molecular ion, as compared with a loss of 113 Da for CsA, indicating that an additional oxygen was present in amino acid 1 (a beta-hydroxy amino acid). The \(^{1}H\)-NMR spectra confirmed the modification of amino acid 1, as shown by the shift in the \(\gamma\)CH\(_2\) peak obtained for this amino acid. The latter procedure also indicated that none of the N-CH\(_3\) groups was demethylated, as evidenced by the presence of seven N-CH\(_3\) singlets and four NH doublets. We identified this metabolite as a dihydroxylated metabolite of CsA, either M-8 or M-26, according to the scheme of Maurer et al. (5).

Peak B. The FAB/MS of metabolite peak B indicated a molecular ion species of m/z 1235 (MH\(^+\)), corresponding to an increase of 32 Da over CsA, which is consistent with the addition of two oxygen atoms. The \(^{1}H\)-NMR spectra con-

Fig. 1. 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. CsA is eluted with a retention time of approximately 100 min (not shown)
confirmed the modification at amino acid 1. We identified this metabolite as a dihydroxylated metabolite of CsA, either M-8 or M-26.

**Peak C.** The protonated molecular ion was at m/z 1205 (MH⁺), which is a 2-Da increase over CsA. We considered this to be due to the addition of one oxygen (+16 Da) and loss of one methyl group (-14 Da). The presence of a fragment with an m/z of 1092 indicated that there was no modification on the side chain of amino acid 1. This was confirmed by ¹H-NMR, in which no shift in the nCH₃ peak of amino acid 1 was observed. A loss of one of the seven N-CH₃ singlets corresponding to amino acid 4 with the presence of five NH doublets indicated that demethylation had occurred at amino acid 4. We identified this compound as a hydroxylated demethylated metabolite of CsA, tentatively M-13, according to the scheme of Maurer et al. (5).

**Peak D.** The protonated molecular ion was at m/z 1219 (MH⁺), corresponding to a 16-Da increase over CsA, attributed to the addition of one oxygen group. The ¹H-NMR and ¹³C-NMR indicated that the modification occurred at amino acid 1. This metabolite was identified as M-17. The FAB/MS, ¹H-NMR, and ¹³C-NMR spectra for this metabolite are shown in Figures 2 and 3.

**Peak E.** The protonated molecular ion was at m/z 1221 (MH⁺), which indicates an 18-Da increase over CsA, considered to be due to the addition of one oxygen (+16 Da) and the addition of two hydrogen molecules (+2 Da). The presence of a 1090-Da fragment indicates that an additional oxygen and hydrogen were contained in amino acid 1. The ¹H-NMR confirmed the modification on amino acid 1. The latter procedure also indicated that there had been no demethylation. We identified this compound as a hydroxylated methylated metabolite of CsA in which the double bond of amino acid 1 is saturated.

**Peak F.** The protonated molecular ion was at m/z 1219 (MH⁺), which is a 16-Da increase over CsA. The presence of a 1106-Da fragment indicated that no modification had occurred on amino acid 1. The ¹H-NMR confirmed this finding. We identified this compound as a monohydroxylated metabolite, tentatively M-1, according to the scheme of Maurer et al. (5).

**Peak G.** The protonated molecular ion was at m/z 1219 (MH⁺), a 16-Da increase over CsA, which we ascribed to the addition of one oxygen group. The presence of a 1090-Da fragment indicated that the oxygen was contained in amino acid 1. The shift of the nCH₃ peak in the ¹H-NMR spectra confirms a modification of amino acid 1. We tentatively identified this compound as M-18 according to the scheme of Maurer et al. (5).

**Peak H.** The protonated molecular ion was at m/z 1189 (MH⁺), a 14-Da decrease from CsA, which we ascribed to the loss of one methyl group (-14 Da). A fragment of 1076 Da was identified, indicating no modification to amino acid 1. The ¹H-NMR indicated the loss of one of the N-CH₃ singlets corresponding to amino acid 4, with an additional NH doublet being observed. We identified this compound as M-21 (5). The FAB/MS and ¹H-NMR spectra of this metabolite are shown in Figure 4.

**Immunosuppressive Activity**

We tested the immunosuppressive activity of the CsA
metabolites on three separate occasions, using primary (1°) and secondary (2°) mixed-lymphocyte cultures as well as phytohemagglutinin-stimulated cultures. Table 1 shows the concentrations of the metabolites that result in 50% inhibition of \(^{3}H\) thymidine uptake (IC\(_{50}\)) in all three test systems. The potency ratios, defined as the ratio of IC\(_{50}\) of metabolites to that of CsA, are also shown. The IC\(_{50}\) of CsA as well as its metabolites varies from system to system. Significantly more CsA and metabolite was 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 testing systems. All the metabolites tested have immunosuppressive activity <10% of CsA (potency ratio < 0.10) except M-17 and M-1, which exhibit potency ratios of 0.16 and 0.14, respectively, in a 2° MLC system. In contrast, M-1, M-17, and M-21 exhibit similar potencies in the 1° MLC system, whereas M-1 is the most immunosuppressive in a phytohemagglutinin mitogen-stimulated system.

**Discussion**

This study was designed to assess the in vitro immunosuppressive effects of the various CsA metabolites that appear in urine from treated renal-transplant patients. A prerequisite for the study was the procurement of CsA metabolites with known structure and purity. We isolated eight metabolites from the urine of renal-transplant recipients. We assessed the structure and purity of all metabolite fractions by FAB/MS and \(^{1}H\)-NMR, as well as \(^{13}C\)-NMR for one fraction (peak D). We were able to assign the structure of the compounds corresponding to peak C (M-13), peak D (M-17), and peak H (M-21). The structures of the other metabolites were tentatively identified according to the scheme of Maurer et al. (5). Definitive structural identification of the remaining metabolites will be possible once sufficient quantities have been isolated to permit analysis of all metabolites by \(^{13}C\)-NMR. All metabolite fractions were assessed to be free of contamination (>99% pure). The structural identity of the metabolites agrees with the recent report of Wang et al. (6), who studied metabolites isolated from bile of transplant recipients, using FAB/MS and \(^{1}H\)-NMR. We also detected the presence of a novel monohydroxylated metabolite first reported in this latter study in which the double bond in amino acid 1 was saturated (6).

We tested the immunosuppressive activities of the metabolites simultaneously in three separate in vitro assay systems. We found the immunosuppressive activities of the metabolites to vary among the assay systems, with most of the metabolites having immunosuppressive activity <10% of that of CsA. This is consistent with the results of Ryffel et al. (11) and Wallemacq et al. (4), who found the metabolites tested, with the exception of M-17, to be 10% as active as CsA. M-17 was found to have immunosuppressive activity of approximately 25% (11) and 50% (4) of that of CsA in the two studies. In contrast, Hartman and Jardine (13) and

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**Table 1. Immunosuppressive Activity of Some CsA Metabolites Compared**

<table>
<thead>
<tr>
<th>Metabolite peak</th>
<th>Modification</th>
<th>1° MLC IC(_{50}) [(\mu g/L)]</th>
<th>2° MLC IC(_{50}) [(\mu g/L)]</th>
<th>Phyt. hemagglutinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Dihydroxylated (M-8 or M-26)</td>
<td>&gt;2000 ((&lt;0.007))</td>
<td>8933 ± 1508 (0.001)</td>
<td>&gt;20 000 (&lt;0.026)</td>
</tr>
<tr>
<td>B</td>
<td>Dihydroxylated (M-8 or M-26)</td>
<td>&gt;2000 ((&lt;0.001))</td>
<td>7270 ± 870 (0.002)</td>
<td>&gt;20 000 (&lt;0.026)</td>
</tr>
<tr>
<td>C</td>
<td>Hydroxylated demethylated (M-13)</td>
<td>1667 ± 189 (0.009)</td>
<td>7250 ± 2478 (0.002)</td>
<td>&gt;20 000 (&lt;0.026)</td>
</tr>
<tr>
<td>D</td>
<td>Hydroxylated (M-17)</td>
<td>170 ± 7 (0.089)</td>
<td>83 ± 17 (0.16)</td>
<td>20 700 ± 3600 (0.025)</td>
</tr>
<tr>
<td>E</td>
<td>Hydroxylated saturated amino acid 1</td>
<td>335 ± 81 (0.042)</td>
<td>527 ± 107 (0.02)</td>
<td>11 200 ± 4200 (0.046)</td>
</tr>
<tr>
<td>F</td>
<td>Hydroxylated (M-1)</td>
<td>157 ± 19 (0.095)</td>
<td>90 ± 24 (0.14)</td>
<td>5630 ± 120 (0.092)</td>
</tr>
<tr>
<td>G</td>
<td>Hydroxylated (M-18)</td>
<td>1500 ± 400 (0.01)</td>
<td>520 ± 106 (0.025)</td>
<td>11 900 ± 2400 (0.044)</td>
</tr>
<tr>
<td>H</td>
<td>Demethylated (M-21)</td>
<td>183 ± 12 (0.082)</td>
<td>367 ± 26 (0.035)</td>
<td>7300 ± 2400 (0.071)</td>
</tr>
<tr>
<td>CsA</td>
<td></td>
<td>15 ± 3 (1.0)</td>
<td>13 ± 2 (1.0)</td>
<td>520 ± 100 (1.0)</td>
</tr>
</tbody>
</table>

* 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.
Schlitt et al. (12) found the metabolites to possess minor immunosuppressive activity. Freed et al. (16) 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. (10), who used secondary proliferative responses of cloned allogeneic T cells and transplant-recipient-induced lymphocytes. A recent report of a novel CsA metabolite (metabolite E) indicated that this compound had an immunosuppressive activity about 80% that of CsA (15). These discrepancies may be due to differences in the purity of the metabolite fractions used as well as to differences among the assays used to assess immunosuppressive activity. In some instances, IC_{50} values or potency ratios for direct comparison of the metabolites and CsA are not reported (9, 10). The present study, in which standardized techniques for metabolite isolation and identification as well as testing of immunosuppressive activity were used, may provide an insight into the matter. Nevertheless, what is consistent among the studies is that metabolites with single modifications—M-17, M-1 (hydroxylation), or M-21 (dimethylation)—possess the most immunosuppressive activity, and that metabolites with two or more modifications possess substantially less.

The overall contribution of CsA metabolites to immunosuppressive activity in vitro still remains to be determined. M-17, which we and others have found to be the most immunosuppressive of all CsA metabolites (3, 4, 9–16), is present in concentrations exceeding those of CsA in the blood of kidney-, liver-, and heart-transplant patients (7, 8). The concentrations of this and other metabolites in tissues have been shown to be severalfold higher than that of CsA itself (7, 8). In liver-transplant patients where the metabolite/CsA ratios are higher, a better correlation with clinical responses was observed when both components were monitored in whole blood (20). The Task Force on CsA Monitoring recommends (21) that CsA should be monitored in whole blood with an assay specific for the parent compound, and that if any metabolites were shown to be active, specific assays for them should be instituted. Although we and others have shown that CsA metabolites, in particular M-17, possess immunosuppressive activity, the requirement for its routine monitoring in cases such as renal-transplant patients, where the metabolite/parent CsA ratio is relatively constant (1), does not appear to be warranted.

In other cases, such as in heart- and liver-transplant recipients, where higher and more variable metabolite concentrations are encountered (1), more clinical data are required before the need for routine clinical monitoring of metabolites can be ascertained.

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References

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