Is Cyclophilin Involved in the Immunosuppressive and Nephrotoxic Mechanism of Action of Cyclosporin A?

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Summary

In this report we have approached two questions relating to the mechanism of action of cyclosporin A (CsA). First, we address whether the major cytosolic protein for CsA, cyclophilin, is directly involved in mediating the immunosuppressive activity of this drug, and, in particular, whether inhibition of this protein's peptidyl-prolyl *cis-trans* isomerase (PPIase) activity results in inhibition of murine T cell activation. Second, we ask whether the nephrotoxicity observed with CsA is related to inhibition of PPIase-dependent pathways in cells other than lymphocytes. Using a series of 61 cyclosporin analogues, we generally found a good correlation between cyclophilin binding and immunosuppressive activity for the majority of analogues analyzed. However, a number of compounds of distinct structural classes were found that could interact with cyclophilin but were much less immunosuppressive than expected. The inability of these analogues to inhibit lymphocyte activation could not be explained by their failure to enter the cell and bind to cyclophilin under the conditions used in the cellular assays. Surprisingly, a nonimmunosuppressive analogue, MeAla-6, which bound well to cyclophilin and was active as a PPIase inhibitor, did not induce renal pathology in vivo. Furthermore, another analogue, $MeBm_2t$, which was immunosuppressive in vitro, possessed little or no activity as a PPIase inhibitor. These findings pose serious questions concerning a direct role of cyclosporin in mediating CsA's immunosuppressive and nephrotoxic activities. In addition, they raise doubts about whether PPIase has a direct function in lymphocyte signal transduction.

Cyclosporin A (CsA)[1] has been widely used for over a decade for the treatment of allograft rejection and graft-vs.-host disease and is currently the treatment of choice for these clinical conditions. Despite CsA's widespread utility, patients treated with this immunosuppressive drug experience significant side effects, including nephrotoxicity, hepatotoxicity, CNS disturbances, hirsutism, and gingival hyperplasia (reviewed in reference 1). Nephrotoxicity, in particular, has limited CsA's promising therapeutic effects in a number of autoimmune disorders (1, 2). While it is clear that the discovery of a safer analogue would greatly expand the clinical utility of this powerful class of immunosuppressive agents, a better understanding of the relationship between the mechanism(s) that cause immunosuppression and nephrotoxicity are needed before such drugs can be developed in a rational manner.

It is well established that CsA inhibits T cell activation by blocking the transcription of a family of early activation genes, including those of the principal T cell growth factors, IL-2 and IL-4 (3–5). However, we and others have concluded that CsA does not act directly at the transcriptional level, but rather inhibits an earlier event in lymphocyte signal transduction associated with activation pathways that cause a rise intracellular calcium (6–10).

A number of molecules have been proposed as the biochemical site of CsA's action in these events. Cyclophilin, dis-

[1] *Abbreviations used in this paper:* BUN, blood urea nitrogen; CsA, cyclosporin A; FKBP, FK-506 binding protein; PPIase, peptidyl-prolyl *cis-trans* isomerase.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/03/0619/10 $2.00
Volume 173 March 1991 619–628

covered by Handschumacher et al. (11) in 1984, is a ubiquitous and abundant cytosolic protein that is highly conserved among eukaryotic organisms, and is responsible for the uptake of CsA into the cell. Several observations support the hypothesis that cyclophilin is involved in CsA's mechanism of action. First, the specificity of cyclophilin binding has been examined with a limited set of cyclosporin analogs, and, with this narrow series, immunosuppressive activity was found to correlate with cyclophilin binding (12). Second, cyclophilin was recently discovered to catalyze the *cis-trans* isomerization of peptidyl proline bonds (13, 14). The ability of CsA to inhibit this enzymatic activity could, in theory, interfere with lymphocyte activation by hindering conformational changes in transcriptional factors, protein kinases, or ion channels. The discovery that the Drosophila *nina*A mutant, which is defective in the conversion of opsin to rhodopsin, has a mutation in a cyclophilin-like gene (15, 16) illustrates how this molecule may be involved in a signal transduction pathway. Recently, Tropschug et al. (17) has shown that yeast and Neurospora that have been selected for CsA resistance possess defects in their cyclophilin genes. Further support for the involvement of cyclophilin in CsA's action has come from the unexpected discovery that the major cytosolic binding protein for the immunosuppressant FK-506, FKBP, has a similar enzymatic activity (18, 19). These correlative observations suggest a critical role for cyclophilin in the mechanism of action of CsA, but in mammalian cells no biochemical or genetic data exist to provide a direct link. Thus, although it is clear that cyclophilin is a major cytosolic receptor for CsA and is responsible for the accumulation of the compound within the cell, the question of whether cyclophilin is the mediator of CsA's immunosuppressive and/or nephrotoxic activities has not been clearly resolved.

In this report we have taken a pharmacologic approach to address this question. We have compared cyclophilin binding, in vitro and in vivo immunosuppressive activity, peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, and in vivo nephrotoxicity for a more extensive and structurally diverse set of cyclosporin analogues than previously investigated. 61 compounds were prepared, including substitution at the various amino acid residues of the peptide cycle and sidechain modifications of the unique amino acid residue at position 1. Preliminary reports with some of these compounds have previously appeared (20). Overall, we found a good correlation between cyclophilin binding and immunosuppressive activity for the majority of analogues analyzed. On the other hand, a number of compounds of distinct structural classes were found that could interact with cyclophilin but were much less immunosuppressive than expected. The inability of these analogues to inhibit lymphocyte activation could not be explained by their failure to enter the cell and bind to cyclophilin under the conditions used in the cellular assays. Surprisingly, the nonimmunosuppressive analogue MeAla-6, which bound well to cyclophilin, was active as a PPIase inhibitor, but did not induce renal pathology in vivo. Furthermore, another analog, $MeBm_2t$, that was immunosuppressive in vitro, bound weakly to cyclophilin and possessed little activity as a PPIase inhibitor. These findings pose serious questions concerning a direct role of cyclophilin in mediating CsA's immunosuppressive and nephrotoxic activities. In addition, they raise doubts about whether PPIase has a direct function in lymphocyte signal transduction.

Materials and Methods

CsA Analogues. [^{3}H]dihydro cyclosporin was made by catalytic reduction of the MeBmt double bond in CsA with tritium gas by New England Nuclear (Boston, MA) using conditions and CsA supplied by our laboratories.

The cyclosporin analogues included in this study were obtained either by totally synthetic methods or semi-synthetic modification of CsA. Crystalline CsA was isolated from Sandimmune Oral Solution by flash chromatography on silica gel using dichloromethane-methanol mixtures to elute CsA. Fractions containing UV-light-positive material were combined and evaporated. Crystallization was affected with acetone-hexane (92% recovery). The totally synthetic analogues were prepared via cyclization of the appropriate unprotected linear undecapeptide using the mixed phosphonic anhydride method (21), essentially as described by Wenger (22). The precursor tetrapeptide and heptapeptide fragments were derived using a combination of the mixed pivalic anhydride method modified for *N*-methylated amino acid derivatives (23) and the BOP-Cl method (24, 25). Where applicable, use of the BOP-Cl coupling reagent afforded higher yields under more moderate conditions in shorter reaction times. Coupling of the partially protected tetrapeptide with the MeBmt-linked heptapeptide was effected using the Castro (BOP) reagent (22). The synthesis of $MeBm_2t$ cyclosporin has been published (26).

MeBmt was synthesized by modification of the Wenger procedure (27) starting from (*R*,*R*)-(+)-diethyl tartrate and resulting in improvement in the overall yield from 6 to 8%. The fluorinated amino acids employed in the total synthesis of analogues containing a fluoro-substituted amino acid residue at the 2, 5, or 11 positions were provided by Dr. J. Kollonitsch and Mr. L. Perkins of the Merck, Sharp and Dohme Research Laboratories, Basic Chemistry Dept. Structural assignments were made with the aid of high-field proton and carbon-13 NMR spectroscopy and fast atom bombardment (FAB) mass spectroscopy. Preparative details, along with characterization data, will be reported elsewhere.

Proliferation Assays. Mouse splenic T cells were isolated by nylon wool column separation, washed twice, and resuspended at 10^6 cells/ml in culture medium made up of RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated FCS (Flow Laboratories, McLean, VA), 2 mM glutamine, 1 mM sodium pyruvate, 2×10^{-5} M 2-ME, and 50 μg/ml gentamycin. Cell suspensions were seeded (100 μl/well) into flat-bottomed 96-well microplates (Costar, Cambridge, MA). Various dilutions of the compound under test, in parallel with a standard range of CsA concentrations, were added to the wells in triplicates and the cultures received a mixture of ionomycin (250 ng/ml) and PMA (10 ng/ml). Cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2-95% air and pulsed with [^{3}H]TdR (2 μCi/well) for the last 4 h. Cells were harvested on fiber glass filters and the incorporated radioactivity was measured in a Betaplate liquid scintillation spectrometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

In the experiments to test for antagonist activity of CsA analogues, the cells first received various concentrations of MeAla-6 and were incubated for 3 h at 37°C. Various concentrations of CsA were then added and the cultures activated with ionomycin + PMA, as described above.

Cyclophilin Binding Assays. Cyclophilin was purified from calf thymus according to Harding et al. (28). In all experiments described, binding assays were performed with the major (pI 8.6) species of cyclophilin; analogues that showed a discrepancy between immunosuppression and binding to the major isoform were assayed against the minor (pI 6.8) species of cyclophilin. No differences between the two isoforms were observed (data not shown). A modification of the LH-20 binding assay described by Handschumacher et al. (11) was used for competitive binding studies. Compounds were dissolved in ethanol and diluted in 20 mM sodium phosphate + 0.1% Tween-80, pH 7.2, in 100 μl. 50 μl of cyclophilin at 10–20 μg/ml was added + 50 μl of [^{3}H]dihydro cyclosporin (0.6 μg/ml at 4 μCi/μg). After mixing, bound [^{3}H]dihydro cyclosporin was separated from free by passing over a 2-ml micro-column of LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ). The column was eluted with 500 μl of 20 mM sodium phosphate and the void volume counted in Aquasol (Dupont, Boston, MA) in a Beckman Instruments, Inc. (Palo Alto, CA) LS 9000 scintillation counter.

The binding results are reported as the IC_{50} of percent inhibition of binding, calculated as follows: 100 × [100 − (cpm of competitor − cpm of nonspecific binding)/(cpm of maximal bound − cpm of nonspecific binding)].

Nonspecific binding was determined by binding to cyclophilin in the presence of excess CsA (50 μg/ml). Determinations were made in duplicate and the IC_{50}'s calculated by linear regression on 6–8 doubling dilutions of competitor. Analogues were compared to a CsA standard which was run in each experiment.

Competitive whole cell binding assays were performed as follows. Splenic T cells suspended in RPMI 1640 containing 2% FCS were aliquoted at 10^6 cells/sample in a final volume of 300 μl to which were added 8 nM [^{3}H]dihydro cyclosporin (150 × 10^3 cpm) and various concentrations of cold competitor. The samples were mixed vigorously and incubated for 30 min at 37°C. Cells were then washed once with ice-cold RPMI, spun, resuspended in 100 μl medium, and overlayed on a 10% sucrose solution in Eppendorf microcentrifuge tubes. The tubes were spun at 8,000 rpm for 1 min, frozen, and the tips containing the cell pellets were cut out and counted for incorporated radioactivity.

Proline Isomerase Assays. Cyclophilin PPIase activity was assayed as previously described (14, 18) except that the cyclophilin used (at 3.3 μg/ml) was partially purified through the Matrix Blue A affinity chromatography step (28). Briefly, the PPIase assay used measures the *cis* to *trans* isomerization of the proline-alanine peptide bond in the peptide, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Under equilibrium conditions, ~88% of the peptide is present as the *trans* form and is readily cleaved by chymotrypsin. The remaining 12% of the peptide present in the *cis* form is cleaved upon enzymatic conversion to the *trans* form. The size of conversion was monitored by the change in absorbance at 405 nm due to the release of *p*-nitroaniline. Data points were fit to a simple first-order rate law and the rate constant, k, was determined. K_i values for the inhibition of cyclophilin PPIase activity by CsA and other analogs were determined from the dependence of the first order rate constant, k, on inhibitor concentration using the equation of Williams and Morrison (29) for tight-binding inhibition. A computer program, written by Nancy Thornberry of the Department of Enzymology, Merck, Sharp and Dohme Research Laboratories, was used for this purpose.

Nephrotoxicity Assays. 8–12-wk-old BALB/cJ female mice (The Jackson Laboratory, Bar Harbor, ME) were used. Stock solutions of CsA and analogues were made up at 50 mg/ml in 33% ethanol/67% cremophor (BASF, Parsippany, NJ). Further dilutions were made in saline. Assessment of the nephrotoxic potential of the compounds was done with two different treatment protocols. In the first protocol, referred to as chronic nephrotoxicity assay, the mice received seven daily intraperitoneal injections of various doses of compound or of vehicle alone. Serum, kidney, and liver were collected 24 h after the last injection. In the second protocol, mice were given high doses of compound intraperitoneally for 2 consecutive d, and serum, kidney, and liver were taken 48 h later. Measurement of blood urea nitrogen (BUN) levels in the serum was done on an autoanalyzer (model 203; Gilford Instrument Laboratories, Inc., Oberlin, OH). Histopathologic evaluation of kidney and liver samples was done after standard fixation, sectioning and staining procedures by Dr. Srinivasa Prahalada, Merck, Sharp and Dohme Research Laboratories Safety Assessment (West Point, PA).

Delayed Hypersensitivity Response. CBA/J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a sterile, pathogen-free environment and were at least 10–12 wk of age before immunization. Mice were immunized intravenously with 5 × 10^5 SRBC (Colorado Serum Co., Denver, CO) in 0.5 ml of Dulbecco's PBS (Gibco Laboratories, Grand Island, NY). 72 h after sensitization, mice were challenged by subplantar injection in the hind foot with 5 × 10^8 SRBC in 0.05 ml of PBS. The magnitude of the delayed hypersensitivity response was measured 24 h later with a mercury plethysmograph and the data expressed as μl of footpad swelling. CsA and analogs were administered intravenously beginning with the day of immunization and ending on the day of challenge for a total of four daily doses. All compounds were dissolved in ethanol, diluted in cremophor and then into PBS. The ED_{50} for CsA in this assay is ~7 mg/kg.

Results

Correlation between Cyclophilin Binding and Immunosuppressive Activity. A total of 61 cyclosporin analogs were prepared either by totally synthetic methods or semisynthetic modification of CsA. Close to half of these modifications were made in the MeBmt side chain, an amino acid that is uniquely found in CsA and that has previously been implicated in the immunosuppressive activity of the cyclosporins (12). The remainder of the analogues represent modifications at all the other amino acids within the cyclic structure, with particular emphasis on the 2 position and the 6 position (6 and 7 analogues, respectively).

In Fig. 1 we compare the immunosuppressive activity of these 61 analogues with their ability to compete with [^{3}H]dihydro cyclosporin for binding to cyclophilin. Immunosuppressive activity was assessed by three different methods: (*a*) inhibition of IL-2 production by the T cell hybridoma FS.6; (*b*) inhibition of a proliferative response induced in a murine mixed lymphocyte response; and (*c*) inhibition of murine T cell proliferation stimulated by PMA + ionomycin. Since each of these assays give virtually identical results (R^2's, between 0.89 and 0.93), only the PMA + ionomycin assay results are presented and discussed.

The majority of CsA analogues analyzed show good agreement between cyclophilin binding and immunosuppressive activity, as illustrated in Fig. 1 and summarized in Table 1. However, for 14 compounds, this correlation does not hold;

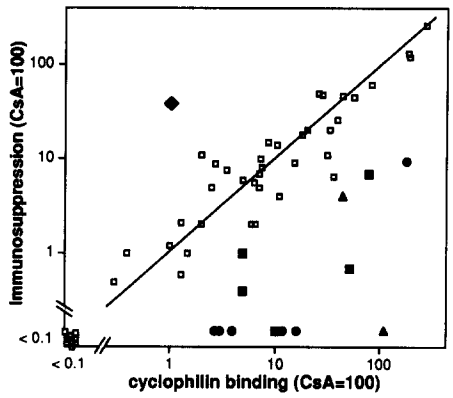

Figure 1. Correlation plot comparing cyclophilin binding and immunosuppressive activities for 61 cyclosporin analogues. All analogues were tested for inhibitory activity in a PMA + ionomycin-induced T cell proliferation assay and for their ability to compete for [^{3}H]dihydro cyclosporin binding to purified calf thymus cyclophilin, with CsA run as a control in each experiment. Results are plotted as the compound's activity relative to CsA. Analogues showing agreement between the two assays (i.e., < five-fold difference between cyclophilin binding and immunosuppression) are plotted as open squares (□). Other classes of compounds are depicted as follows: MeBm$_2$t cyclosporin, (◆); analogues substituted in the 6-position, (■); analogues substituted in the 8 position, (▲); and Bmt derivatives in which the double bond has been eliminated and, in most cases, a sulfur substitution has been made, (●).

these are depicted by solid symbols in Fig. 1. One analogue, MeBm$_2$t cyclosporin, (designated as class III in Table 1 and whose structure is given in Fig. 2), retains immunosuppressive activity but does not compete for [^{3}H]dihydro cyclosporin in the cyclophilin binding assay.

Compounds that retain significant cyclophilin while losing immunosuppressive activity (designated class IV in Table 1) fall into three distinct structural classes. Five of the class IV analogues are modified at the 6 position. Detailed information for this class of compounds is listed in Table 2 and represented by the MeAla-6 derivative in Fig. 2. Two other 6 position analogues, MeNval-6 and MePhe-6, do not show the discrepancy between cyclophilin binding and cell biological readouts. It should be noted that extensive modifications at other positions in the cyclosporin ring, such as at the 2 position, or substitution of alanine at other positions in the ring structure, do not generate compounds that fall into class IV,

Table 1. *Summary of the Correlation Between Cyclophilin Binding and Immunosuppressive Activity*

Class	Cyclophilin binding	Immunosuppression	No. of compounds
I	+	+	37
II	–	–	10
III	–	+	1
IV	+	–	13

* Analogues are designated as showing agreement between the two assays (Class I or II) if they show less than a five fold difference between cyclophilin binding and immunosuppression.

Table 2. *Structure Activity Relationships Among Selected Cyclosporin Analogues*

	Cyclophilin binding (percent of CsA)*	In vitro immunosuppressive activity (percent of CsA)‡
6-position analogues		
$CH_2CH(CH_3)_2$ [CsA]	100	100
CH_3 [MeAla-6]	51	0.4
$CH(CH_3)_2$	5	0.2
CH_2CH_3	78	7
$CH_2CH_2CH_3$	43	45.8
$CH(CH_3)CH_2CH_3$	5	1
CH_2-Phenyl	31	11
CH_3, dihydro MeBmt	10	<0.1
2-position analogues		
CH_2CH_3 [CsA]	100	100
$CH_2CH_2CH_3$ [CsG]	23	45
CH_2SCH_3	2.5	5
$CH_2S(O)CH_3$	<0.1	<0.1
CH_2CHF_2	26	50
CH_2CH_2F	12	8
$CH_2CH_2CH_2F$	2	2
Alanine ring substitutions		
D-MeAla-3	82	61
MeAla-4	6.5	2.5
Ala-5	10.5	14
MeAla-9	7.3	10
MeAla-10	3.5	7.5
MeAla-11	8.5	15
Selected MeBmt modified analogues		
$CH(OH)CH(CH_3)CH_2$ $CH=CH(CH_3)$ [CsA]	100	100
$CH(OH)CH(CH_3)CH_2$ SCH_3 [MeThia-Bmt]	178	9.5
$CH(OH)CH(CH_3)$ $CH_2S(O)CH_3$	2.7	<0.1
$CH(OH)CH(CH_3)$ $CH_2S(O_2)CH_3$	4	<0.1
$CH(OH)CH(CH_3)$ $CH_2CH_2SCH_3$	12	<0.1
$CH(OH)CH(CH_3)$ CH_2S-Phenyl	18	18
$CH(OH)CH(CH_3)$ CH_2COOH	3	<0.1
$CH(OH)CH(CH_3)$ CH_2CH_2OH	16	<0.1

* Cyclophilin competitive binding assay.
‡ PMA + ionomycin-induced murine T cell proliferation assay.

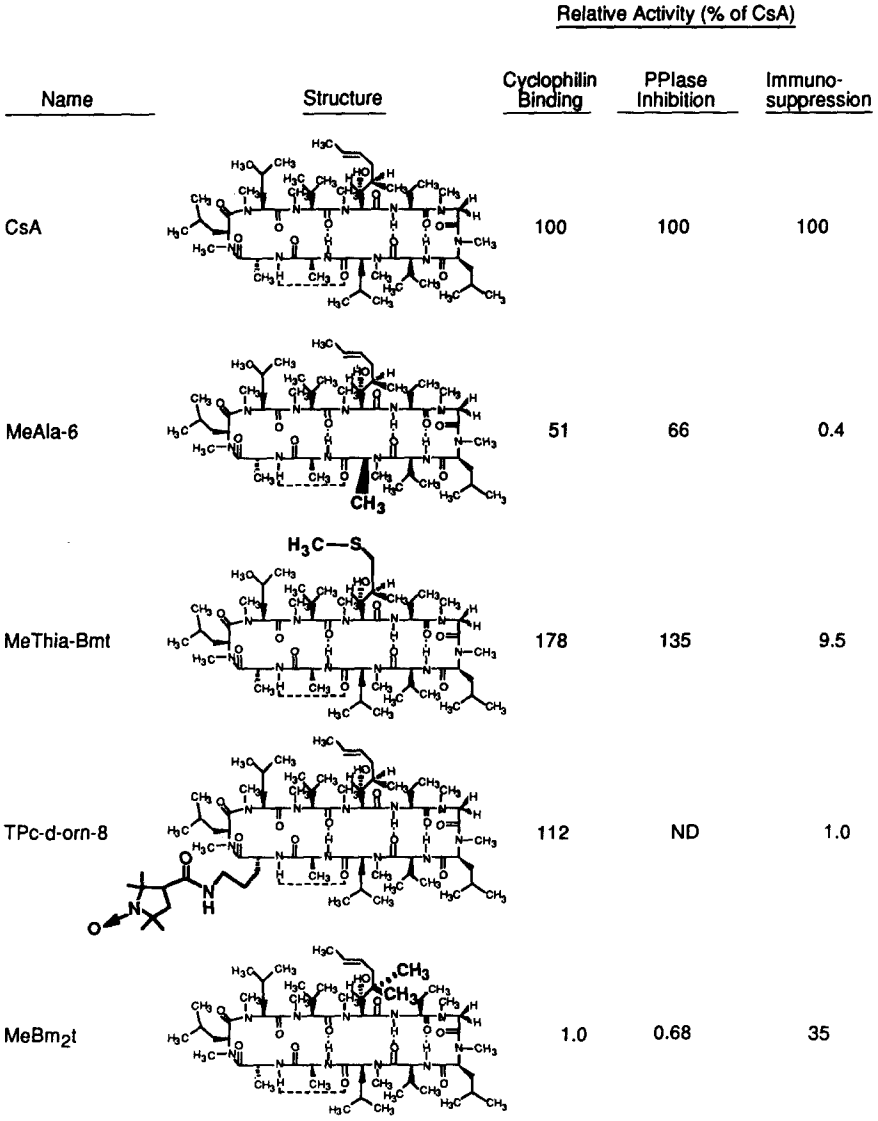

Figure 2. Structures of CsA and representative cyclosporin analogues. Data showing the relative activity of these analogues in the cyclophilin column binding assay, the PMA + ionomycin T cell proliferation assay and cyclophilin peptidyl-prolyl *cis-trans* isomerase (PPIase) assay are also listed in the figure.

as shown in Table 2. Four of the class IV analogs are MeBmt derivatives in which the double bond has been replaced with a sulfur (see Table 2 and example in Fig. 2). All but one sulfur-substituted MeBmt derivative fall into class IV, whereas 20 of 22 other MeBmt modifications fall into either class I or class II. Two analogues substituted at position 8 also fall into class IV. It is evident from this analysis that dissociation of cyclophilin binding and immunosuppression is a property of a distinct set of structural changes within the cyclosporin molecule.

Analysis of Class III and IV Compounds. The significant number of CsA analogues of distinct structural classes that show a discrepancy between cyclophilin binding and immunosuppressive activity calls into question the hypothesis that cyclophilin is the biochemical mediator of this agent's biological action. Therefore, we investigated whether compounds that fell into class III or class IV behave differently in culture than they do in the cell-free cyclophilin binding assay. For example, one reason for a class IV compound to behave in such a manner may be because it is degraded in cell culture or, in some other way, fails to enter the cell and bind to cyclophilin. To address this possibility, we established a whole cell competitive binding assay in which a compound competes with [^{3}H]dihydro cyclosporin for uptake into the lymphocytes used in the biological assay. Merker and Handschumacher (30) previously showed that cellular uptake of CsA at 37°C is due to its interaction with cytosolic cyclophilin. In these experiments, we have focused the majority of our attention on the MeAla-6 analogue (Fig. 2), since it has a large differential between cyclophilin binding (50–70% of CsA) and immunosuppressive activity (0.4% of CsA) and the single class III analogue MeBm$_2$t.

The results of a representative whole cell competitive binding assay with CsA, MeAla-6 and MeBm$_2$t are shown in Fig. 3. The experiment illustrates that MeAla-6 competes with [^{3}H]dihydro cyclosporin uptake almost as well as does CsA. Similar curves are obtained when the competition assay is carried out for 4 h rather than the 1-h incubation shown in Fig. 3 (data not shown). Therefore, since CsA must act within the first few hours after T cell activation (31), the

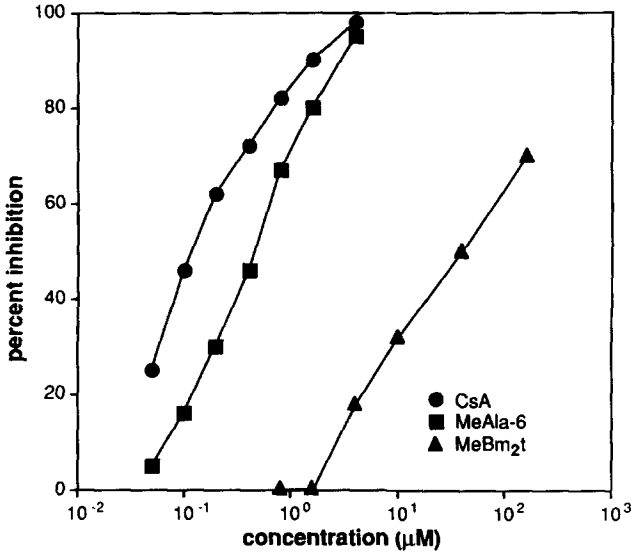

Figure 3. Effect of cyclosporin analogues on the binding of [^{3}H]dihydro cyclosporin to murine T cells. Purified murine T cells were incubated at 37°C with 8 nM (150 × 10^3 cpm) of [^{3}H]dihydro cyclosporin, along with increasing concentrations of CsA (●), MeAla-6 (■), or $MeBm_2t$ (▲). After 60 min, the cells were centrifuged through a layer of 10% sucrose to separate free from cell-bound radioligand and the pellet counted. Data are expressed as the percent inhibition of the control specific binding (85% of total bound radioactivity), determined after subtraction of the radioactivity incorporated in the presence of 2.5 μM CsA.

lack of immunosuppressive activity is not due to degradation or failure to cross the plasma membrane. Indeed, all four class IV compounds that had been determined to bind to cyclophilin in the more conventional biochemical assays were also found to compete in the cellular assay, including one of the sulfur-substituted MeBmt analogues, MeThia-Bmt (Fig. 2). $MeBm_2t$ was a relatively ineffective competitor in this assay, consistent with the results from the cell-free competitive binding assay. These data further suggest that $MeBm_2t$ immunosuppressive activity is not due to unexpected transport or concentration within the cell.

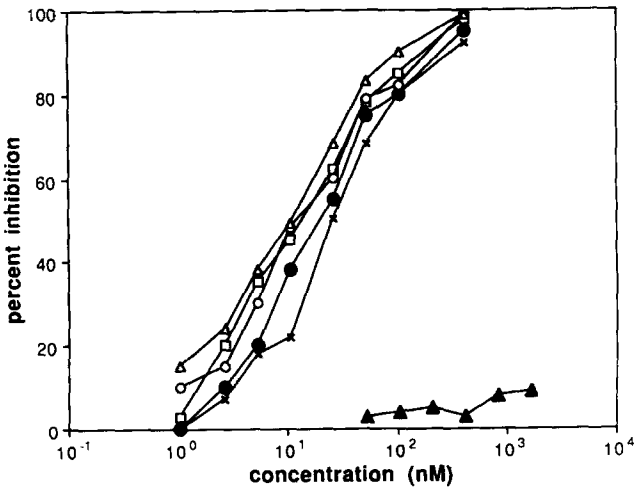

Figure 4. Effect of MeAla-6 on the immunosuppressive activity of CsA. Various concentrations of MeAla-6 or media were added to cultures of murine T cells and incubated for 3 h at 37°C. CsA was then added and the cultures activated with ionomycin + PMA. Final cultures contained CsA alone (●), MeAla-6 alone (▲), or various concentrations of CsA along with MeAla-6 at the following concentrations: 25 nM (□); 100 nM (x); 400 nM (○); 1666 nM (△). Data are represented as the percent inhibition of the control proliferative response.

If cyclophilin is the mediator of CsA's immunosuppressive activity, then we would predict that MeAla-6 should block the biological effects of CsA, since it can enter the cell and displace [^{3}H]dihydro cyclosporin's binding to cyclophilin. Rapamycin, a compound structurally similar to the immunosuppressant FK-506, has been recently shown to block uptake of [^{3}H]dihydro FK-506 and act as a biological antagonist of FK-506 (32). In order to test whether MeAla-6 could antagonize CsA in a similar manner, MeAla-6, at concentrations ranging from 25 to 1,666 nM, was added to murine lymphocytes either alone or in combination with CsA, and the lymphocytes stimulated with PMA + ionomycin. The results of a representative experiment, shown in Fig. 4, demonstrate that MeAla-6 had no effect on the immunosuppressive activity of CsA. The concentration of CsA needed to inhibit 50% of the proliferative response is 21 nM in the control culture and range from 11 to 24 nM in the cultures to which MeAla-6 was added. Additional experiments in which cells were preincubated with MeAla-6 for shorter intervals before addition of CsA or in which alternative readouts (e.g., IL-2 production) or cell populations were employed also failed to demonstrate that MeAla-6 could act as a biological antagonist of CsA (data not shown). It should also be pointed out that at the highest concentration used in these experiments, MeAla-6 is present at an 80-fold excess over the concentration of CsA needed for 50% inhibition, a ratio sufficient to inhibit CsA's binding by 99%.

PPIase Inhibitory Activity of Cyclosporin Analogues. The experiments in the preceding section raise questions about the physiologic role of cyclophilin with respect to its involvement in mediating the immunosuppressive effects of CsA. Perhaps class IV compounds, such as MeAla-6, bind to cyclophilin in a manner that does not inhibit the subsequent biochemical steps in which the protein is involved. Since cyclophilin has been shown to have PPIase activity (13, 14), we next asked whether immunosuppressive activity correlates better with inhibition of enzyme activity than it does with binding. Therefore, selected CsA analogues were assayed for their ability to inhibit cyclophilin's PPIase activity, and the results are shown in Fig. 5. MeAla-6 is comparable to CsA as a PPIase inhibitor, whereas $MeBm_2t$ is a very weak PPIase inhibitor. MeThia-Bmt was somewhat more active than CsA and MeLeu-11 was inactive. The data clearly show that analogues that can effectively compete with [^{3}H]dihydro cyclosporin for binding to cyclophilin can inhibit its PPIase activity, irrespective of the compound's immunosuppressive activity. In particular, MeAla-6, which lacks appreciable immunosuppressive activity, inhibits cyclophilin's PPIase activity, while $MeBm_2t$ is an effective immunosuppressant but is a poor PPIase inhibitor. Thus, for certain structural classes of analogues, binding to cyclophilin and inhibition of PPIase activity are not sufficient to confer immunosuppressive activity.

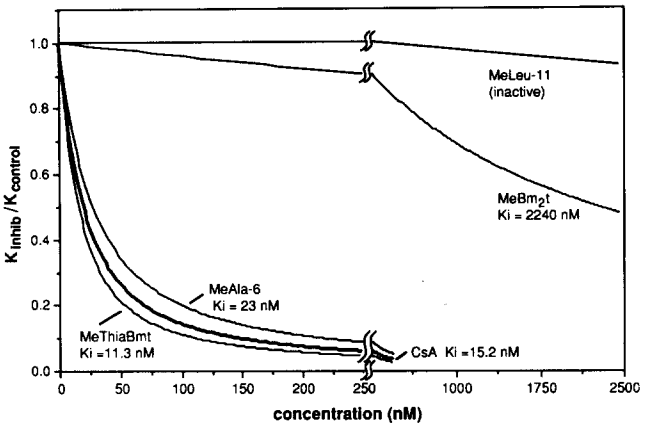

Figure 5. Effects of various cyclosporin analogues on cyclophilin peptidyl-prolyl *cis-trans* isomerase activity. Cyclosporin analogues (1 mg/ml stock solutions in ethanol) were added to the standard PPIase assay (see Materials and Methods) at the concentrations indicated and the rate of isomerization calculated. The data are presented as $K_{inhib}/K_{control}$, where $K_{control}$ is the observed rate constant in the presence of enzyme and in the absence of inhibitor and K_{inhib} is the observed rate constant in the presence of enzyme and inhibitor.

Nephrotoxicity of Cyclosporin Analogues. CsA causes alterations in renal function in a number of experimental animal models, and nephrotoxicity is one of the principal clinical side effects of this drug (1). The most immediate effects of CsA are on renal blood flow (33, 34). Theories put forward to explain this phenomenon include induction of thromboxane (35, 36) or inhibition of prostacyclin release (37) from endothelial cells, a direct effect on vascular smooth muscle contractility (38, 39), an effect of the drug on neurotransmitter release (40), and inhibition of renal microsomal protein chain elongation (41). Using the CsA analogues that differ in their biochemical and immunosuppressive properties, we have chosen to address a different set of questions in this study. First, is the nephrotoxicity of CsA due to its ability to inhibit signal transduction pathways in cell types other than the lymphocyte? Second, does the nephrotoxic potential of a compound correlate with its immunosuppressive activity, with its PPIase inhibitory activity or with some structural feature of the cyclosporin molecule unrelated to these mechanisms?

Three in vivo assays were established to resolve these questions. To determine whether analogues were immunosuppressive in vivo, we assayed selected compounds for their ability to inhibit a delayed hypersensitivity response, and we obtained good agreement between the in vitro and in vivo assays, as shown in Table 3. Next, two assays of nephrotoxicity were established. In the first assay, BALB/c mice were given two intraperitoneal doses of CsA or analogue (50–400 mg/kg) at 24-h intervals; 24 h after the second dose, BUN levels were measured and the kidneys removed for histopathology. Some deaths were recorded in mice treated with 200 mg/kg CsA, and there was modest elevation (30%) of BUN at 100 mg/kg CsA. Microscopic examination of the kidneys in animals treated with 200 mg/kg of CsA revealed evidence of diffuse tubular degeneration characterized by cytoplasmic swelling and vacuolation and dilation of tubular lumina, similar to previous reports of CsA renal histopathology (42). The renal morphology was unremarkable at 100 mg/kg CsA. In the second nephrotoxicity assay, BALB/c mice were treated, by intraperitoneal route, for 7 d with 50–200 mg/kg of compound and killed on day 8 for histology and serum chemistries. While there was little evidence of histopathologic change in mice treated with 50 mg/kg CsA, BUN elevation twofold above control levels was typically observed.

Both assays were performed on the compounds listed in Table 3. For each analogue listed, the severity of nephrotoxicity was equivalent in both assays. The level of renal dysfunction is based on the concentration required to induce his-

Table 3. *Ability of Cyclosporin Analogues to Induce Nephrotoxicity In Vivo Correlates with their Immunosuppressive Activity*

Name	Cyclophilin binding (percent of CsA)*	In vitro immunosuppressive activity (percent of CsA)‡	In vivo immunosuppressive activity (percent of CsA)§	Nephrotoxicity‖
CsA	100	100	100	+ + +
CsG	23	45	50	+ + +
dihydro	36	38	33	+
MeThia-Bmt	178	9.5	23	−
MeAla-6	51	0.4	<10	−
MeLeu-11	<0.1	<0.1	ND	−

* Cyclophilin competitive binding assay.
‡ PMA + ionomycin-induced murine T cell proliferation assay.
§ Murine sheep red blood cell–specific delayed hypersensitivity response.
‖ Induction of nephrotoxicity, as measured in the 2- and 7-d assays described in Materials and Methods. Relative scoring of nephrotoxicity in the 2-d assay was as follows: + + +, pathologic changes at 200 mg/kg; + +, pathologic changes at 400 mg/kg; +, mild pathologic changes at 400 mg/kg; −, no pathologic changes at 400 mg/kg. Relative scoring of nephrotoxicity in the 7-d assay was as follows: + + +, BUN elevation at 50 mg/kg; + +, BUN elevation at 100 mg/kg; +, BUN elevation at 200 mg/kg; −, no BUN elevation at 200 mg/kg.

topathologic changes in the 2-d assay and significant BUN elevation in the 7-d assay. The highest concentrations of drugs tested were 400 mg/kg in the 2-d assay and 200 mg/kg in the 7-d assay. These restrictions were imposed by constraints of compound availability and solubility.

The three immunosuppressive analogs listed in Table 3, CsA, CsG, and dihydro cyclosporin, induce significant nephrotoxicity in these experimental models, whereas the nonimmunosuppressive compounds, MeAla-6 and MeLeu-11, are not nephrotoxic. Another class IV compound, MeThia-Bmt, which binds well to cyclophilin but is only weakly immunosuppressive, does not induce renal pathology. This analogue may have failed to induce renal pathology because the nephrotoxicity assay may not have sufficient sensitivity to pick up such a weakly active compound. Nevertheless, the results in Table 3 demonstrate that immunosuppressive activity, and not cyclophilin binding or PPIase inhibitory activity, determines the ability of CsA analogues to induce nephrotoxicity.

Discussion

In this report we analyze 61 CsA analogues for their ability to inhibit lymphocyte activation, to bind to cyclophilin, to inhibit PPIase activity, and to induce renal pathology. The central question addressed in these experiments concerns the role of cyclophilin in mediating the immunosuppressive and nephrotoxic mechanism(s) of action of CsA. Since its identification by Handschumacher et al. (11) in 1984, cyclophilin has been an attractive biochemical target as the transducer of CsA's action. In contrast to other molecules identified as CsA receptors, such as calmodulin (43, 44) and the prolactin receptor (45), several groups have demonstrated that only immunosuppressive CsA analogs can interact with cyclophilin (11, 12). While it is unclear how a protein as ubiquitous as cyclophilin could mediate CsA's activity in a lymphocyte-selective manner, the hypothesis favoring a critical role for cyclophilin was strengthened by the discovery of an enzymatic activity for this protein (13, 14). Another observation further supporting this notion was the recent finding that the major cytosolic receptor for another immunosuppressive agent, FK-506, is also a PPIase (18, 19). Despite this impressive body of circumstantial evidence, experiments presented in this paper lead us to conclude that the role of cyclophilin in lymphocyte signal transduction remains uncertain.

As shown in previous studies (12), a good correlation was observed between cyclophilin binding and immunosuppressive activity for the vast majority of analogues analyzed. Combined with the observation that the major intracellular receptors for both CsA and FK-506 possess PPIase activity (18, 19), the observed correlation suggests that cyclophilin, or a closely related molecule, is involved in CsA's immunosuppressive mechanism of action.

On the other hand, a number of compounds of distinct structural classes (collectively designated as class IV compounds) were found that could interact with cyclophilin but were much less immunosuppressive than expected. In addition, $MeBm_2t$ was immunosuppressive yet bound weakly to cyclophilin and lacked significant PPIase inhibitory activity. The inability of the class IV analogs to inhibit lymphocyte activation could not be explained by their failure to enter the cell and bind to cyclophilin under the conditions used in the cellular assays. Furthermore, at concentrations of MeAla-6 that should have occupied most the intracellular cyclophilin, this analogue could not be demonstrated to act as an antagonist of CsA's immunosuppressive activity. Thus, the paradox created by the class IV compounds suggests that the relationship, if any, between cyclophilin binding and immunosuppressive activity is not a direct one.

The MeAla-6 and $MeBm_2t$ analogues inhibit cyclophilin's PPIase activity in proportion to their ability to displace [^{3}H]dihydro cyclosporin from the molecule and not in agreement with their immunosuppressive activity. This finding clouds the hypothesis that PPIase has a direct function in lymphocyte signal transduction. One must consider, however, that the PPIase assay currently used employs a synthetic tetrapeptide as substrate which may not accurately reflect the more complex interactions between cyclophilin and polypeptide substrates within the cell. We and others have speculated that PPIases could be involved in signal transduction pathways through alteration of the conformation of transcription factors, ion channels, or protein kinases. Although the fact that the FK-506 binding protein, FKBP, also is a PPIase (inhibitable by its immunosuppressive ligand) (18, 19) must be viewed as more than a fortuitous coincidence, the evidence points to a much more subtle/complex role for these enzymes than previously hypothesized.

What alternatives to a central role for cyclophilin might be envisioned? First, other proteins, perhaps evolutionarily related to cyclophilin, may be involved in CsA's mechanism of action. Southern blotting has revealed a number of genes that crosshybridize with a cyclophilin cDNA probe (46). A gene product genetically related to cyclophilin and perhaps expressed in a lymphocyte-specific fashion could account for the correlation between immunosuppression and cyclophilin binding and for the selectivity of CsA for lymphocyte signal transduction pathways. Such a protein would be expected to bind $MeBm_2t$ but fail to interact with MeAla-6. A second possibility might be that binding to cyclophilin and PPIase inhibition is necessary but not sufficient for immunosuppression. For example, cyclophilin, through its PPIase activity, may alter the conformation of CsA in such a way that it can now bind to a second acceptor molecule, which actually mediates CsA's immunosuppressive effect. Recently, Fesik et al. (47) have shown by isotope filtered NMR methods that CsA does adopt a new conformation when it is bound to human or bovine cyclophilin. The bound conformation of CsA contains a *trans* amide bond between amino acid residues MeLeu-9/MeLeu-10 instead of the *cis* amide bond present in the solution and X-ray conformations. It is possible that the class III analogue $MeBm_2t$ is able to bind efficiently to the immunosuppressive receptor without assistance from cyclophilin. In contrast, an analogue such as MeAla-6 might interact appropriately with cyclophilin but fail to bind to the acceptor protein. Third, cyclophilin, in addition to its role as a PPIase involved in protein folding, may be a subunit of

a larger holoenzyme complex, the function of which is important in lymphocyte activation but only distantly related to its known PPIase activity. Disulfide isomerase, an enzyme important in protein folding, has been documented to function as a subunit in enzymatic activities unrelated to its housekeeping role (48).

The observation that induction of nephrotoxicity correlates with the immunosuppressive activity of the CsA analogues tested has important clinical and mechanistic implications. The results suggest that it will be difficult to discover nonnephrotoxic CsA analogues that retain significant immunosuppressive activity. Segregation of the two activities may still be possible by altering a compound's distribution or metabolism. From a mechanistic perspective, the data imply that nephrotoxicity is due to similarities in signal transduction pathways between the lymphocyte and the cells involved in the renal pathophysiology. The principal side effect of CsA is clearly not due to inhibition of PPIase activity in other cell types. The question of whether identical molecules mediate nephrotoxicity and immunosuppression or whether the side effects are due to crosstalk between related pathways remains to be determined.

The remarkable similarities between CsA and FK-506 with respect to their cellular and biochemical mechanism of action provide an important opportunity to better understand how these molecules interfere with lymphocyte activation and, in this way, gain insight into the biochemical processes that mediate signal transduction. Both CsA and FK-506 selectively inhibit lymphocyte activation pathways that are associated with a rise in intracellular calcium (6–10). The events inhibited by these agents appear to be distal to membrane-associated processes, such as calcium flux, phosphoinositide generation, and phosphorylation events (49, 50), but proximal to the transcriptional events regulating lymphokine gene expression. Identification of the biochemical steps critical to the action of these immunosuppressants should take into account these observations. The studies described in this report underscore the complexity of these intermediate steps in the transduction process. Experiments with FKBP suggest a level of complexity similar to that observed with cyclophilin. In particular, rapamycin, a molecule structurally related to FK-506, binds to FKBP and inhibits its PPIase activity, but does not inhibit IL-2 gene transcription and, indeed, acts as an antagonist of FK-506's immunosuppressive activity (reference 32 and J. Siekierka, R. Harrison, C. S. Lin, S. H. Y. Hung, and N. H. Sigal, manuscript submitted for publication). Although we do not, as yet, have a sufficient understanding to reconcile such observations, it is hoped that the insights gained will lead to the discovery and development of more potent and selective immunosuppressive agents.

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Received for publication 14 August 1990 and in revised form 9 November 1990.

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