Branches on the α-C Atom of Cyclosporin A Residue 3 Result in Direct Calcineurin Inhibition and Rapid Cyclophilin 18 Binding

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The immunosuppressive drug cyclosporin A (CsA) is a bifunctional molecule. It directly inhibits the peptidyl-prolyl *cis*/*trans* isomerase (EC number 5.2.1.8) (PPIase) cyclophilin 18 (Cyp18), while the resulting Cyp18–CsA binary complex targets the serine/threonine phosphatase (EC number 3.1.3.3) calcineurin (CaN) through a gain-of-function mechanism.[1] Whereas CaN inhibition is thought to be the main contribution of CsA in immunosuppression, many recent findings have also indicated essential roles of Cyp18 in various cellular events.[2] For example, Cyp18 is required for the HIV-1 life cycle.[3] To dissect the numerous biological effects involved in CsA treatment and distinguish the Cyp18 and CaN inhibition, the design of CsA derivatives that inhibit CaN specifically would shed new light in this field.

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Because only the Cyp18–CsA complex, but neither Cyp18 nor CsA alone inhibits CaN, a structural comparison of the free CsA molecule and its complex form with Cyp18 provided us with some clues for the design of monospecific CsA derivatives.[4,5] In its free form (Figure 1), CsA has four intramolecular H bonds and one *cis* amide bond between MeLeu9 and MeLeu10. Furthermore, a β-II′ turn involving residues 2–5 has been observed.[6] Induced by Cyp18 binding, CsA undergoes a dramatic conformational change; namely, all peptide bonds become *trans* and the four intramolecular H bonds disappear.[7,8] We hypothesize that this major alternation of CsA conformation is essential for CaN inhibition by the Cyp18–CsA. The questions raised are whether a chemical modification of CsA could mimic the effect of Cyp18 binding and lead to a conformation similar to that in the Cyp18–CsA complex, and whether the resulting CsA derivative alone could inhibit CaN without Cyp18. Early efforts to mimic the Cyp18-bound CsA structure, by adding conformational restraints through introducing an intramolecular bridge based on a computational molecule design,[9] did not result in direct CaN inhibitor, but increased the inhibition of Cyp18 PPIase activity and CaN inhibition by a Cyp18–CsA derivative complex.

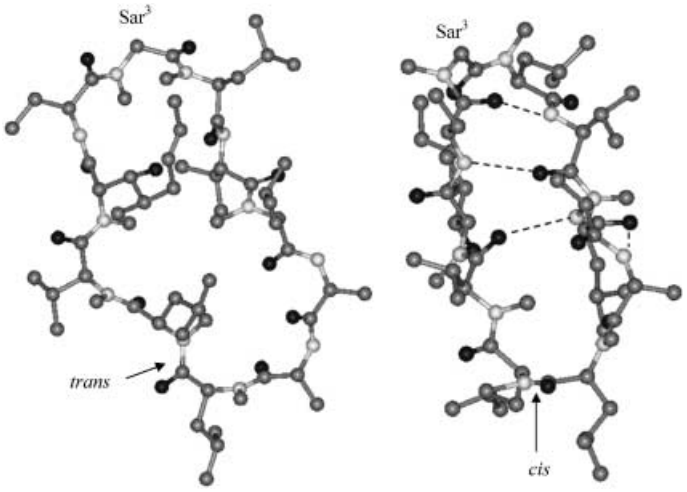
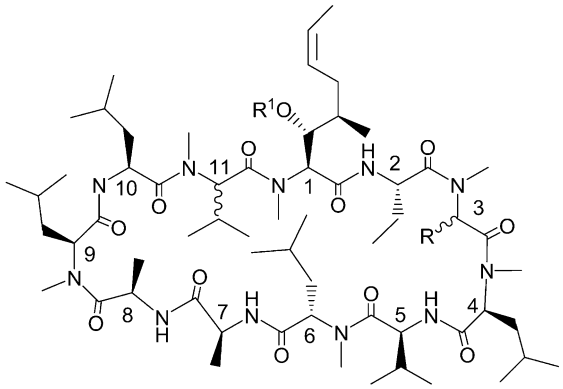


Figure 1. *Conformations of CsA's complex with Cyp18 (left) and unbound form (right). The H-bonds are indicated as dashes. The peptide bonds between MeLeu9 and MeLeu10 are indicated with arrows.*

To achieve the desired conformational switch in CsA, we considered sarcosine 3 (Sar^3, Sar = *N*-methyl glycine) as the position of choice for modification for the following reason: in free CsA, Sar^3 is at the *i*+1 position of the β-II′ turn. In the Ramachandram diagram, among the 20 amino acids, glycine is the only residue accepted for a β-II′ turn at the *i*+1 position. Therefore, we proposed that Sar^3 is required for forming the β-II′ turn in the uncomplexed ligand, and that the turn structure could be impaired through substitution of the Sar^3 α-C atom. A previous NMR study[10] of a water soluble CsA derivative with modified residues 3 and 8 argued that the structure of free CsA in aqueous solution is similar to that in the complex with Cyp18; however, an influence of Sar^3 modification on the structure of the unbound derivative could also be accounted for.

The synthesis of Sar^3 α-C-substituted CsA derivatives had been reported by Seebach's group.[11] The use of a slightly altered synthesis procedure enabled the preparation of a series of CsA analogues listed in Scheme 1.



Scheme 1. *Chemical structures of CsA and its derivatives. CsA: R = H; R^1 = H; L-MeVal on residue 11; [MeSar]^3 CsA: R = Me(R); R^1 = H; L-MeVal on residue 11; [DMeSar]^3 CsA: R = Me(S); R^1 = H; L-MeVal on residue 11; [MsSar]^3 CsA: R = SMe(R); R^1 = H; L-MeVal on residue 11; [DMsSar]^3 CsA: R = SMe(S); R^1 = H; L-MeVal on residue 11; [AcMeBmt]^1[MeSar]^3 CsA: R = Me(R); R^1 = Ac; L-MeVal on residue 11; CsH: R = H; R^1 = H; D-MeVal on residue 11; [MeSar]^3 CsH: R = Me(R); R^1 = H; D-MeVal on residue 11; [DMeSar]^3 CsH: R = Me(S); R^1 = H; D-MeVal on residue 11.*

Due to the multiple conformations of CsA in high polar solvents,[12] CD spectroscopy is the analysis method of choice to investigate the overall structure, especially the formation of the β-II′ turn. We compared the CD spectrum of CsA with its derivatives and found that Sar^3 α-C substitution impaired the β-II′ turn structure significantly (Figure 2). Under the same conditions, the bands corresponding to the β-II′ turn at around 232 nm[13] of [MeSar]^3 CsA and [DMeSar]^3 CsA decreased by about 50%, as compared to CsA. The differential CD spectrum of free Cyp18 and the Cyp18–CsA complex might reflect the CsA structure in the complex with Cyp18 because Cyp18 undergoes only a minor conformational change upon CsA binding.[14] As shown in the insert of Figure 2, the differential spectrum gives a weak band at around 232 nm similar to that of [MeSar]^3 CsA.

CsA is a slow-binding inhibitor of Cyp18, as demonstrated by the time-dependent inhibition of Cyp18 PPIase activities.[15,16] The conformational change of CsA during Cyp18 binding, especially the *cis* to *trans* isomerization of the MeLeu9–MeLeu10 bond, is responsible for the slow kinetics. To estimate whether Sar^3 α-C modifications influence the dynamics of the drug molecule, the binding kinetics of CsA, [MeSar]^3 CsA and [MsSar]^3 CsA to Cyp18 were studied. Cyp18 contains a single tryptophan at position 121 whose fluorescence intensity increases upon binding to CsA.[17] Based on this behaviour, a real-time observation of the binding kinetics of CsA and its analogues to Cyp18 can be achieved by using fluorescence spectroscopy. As shown in Figure 3, the binding of CsA to Cyp18 can be divided into three phases. The first one is very fast and so could not be resolved with manual mixing proce-

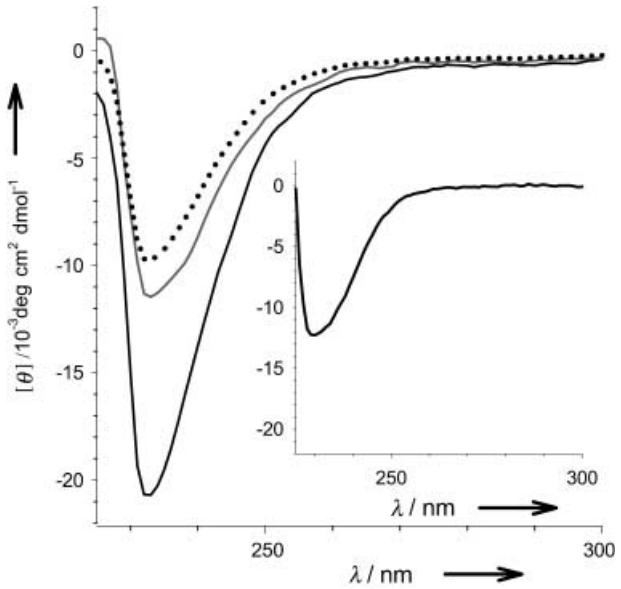


Figure 2. CD spectra of CsA and its derivatives recorded at 10°C. The concentrations of CsA (solid line), [MeSar]^3 CsA (grey) and [DMeSar]^3 CsA (dots) are 0.1 mM in DMSO/water (10:90). The insert represents the differential spectrum of free Cyp18 and the Cyp18–CsA complex. The CD spectra of Cyp18 (0.1 mM) and the Cyp18–CsA complex (0.1 mM Cyp18 and 0.1 mM CsA) were measured in 35 mM Hepes buffer (pH 7.8) at 10°C.

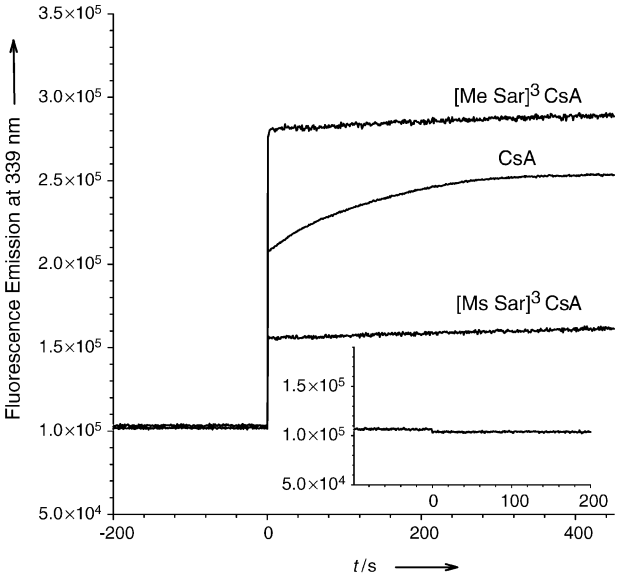


Figure 3. Fluorescence time courses of Cyp18 upon binding to CsA and its derivatives. The intrinsic fluorescence of Cyp18 was monitored at 5°C in 35 mM Hepes buffer (pH 7.8). The concentration of Cyp18 was 8 μM. The CsA derivatives were added to final concentrations of 8.8 μM at time zero. The excitation wavelength was 280 nm with a spectral bandwidth of 3 nm. Emission was detected at 339 nm, also with a spectral bandwidth of 3 nm. The insert represents the fluorescence course upon addition of DMSO at time zero as control.

dures. This fast phase may result from a fraction of Cyp18-active CsA isomer in DMSO.[15] The value of k_{obs} for the slower second phase is $7.6 \pm 0.02 \times 10^{-3}$ s^{-1} and corresponds to the *cis*-to-*trans* isomerization of the MeLeu9–10 peptide bond and the following formation of the tightly bound Cyp18–CsA complex. The third phase of binding is extremely slow, and therefore could not be evaluated exactly. These results are in good agreement with former time-dependent Cyp18 PPIase-activity-inhibition assays;[15,16] this shows that the second phase represents the slow inhibition kinetics of Cyp18 by CsA. The fluorescence time courses of Cyp18 upon binding to [MeSar]^3 CsA and [MsSar]^3 CsA were different from that of Cyp18–CsA binding. In these cases, only two kinetic phases could be observed, the second slow phase was lacking. With impaired CaN-inactive β-II′ turn structures, [MeSar]^3 CsA and [MsSar]^3 CsA appear to be fast-binding inhibitors of Cyp18. In addition to the fast binding kinetics, the fast inhibition kinetics of [MeSar]^3 CsA and [MsSar]^3 CsA to Cyp18 were demonstrated by time-dependent Cyp18 PPIase-inhibition assays (data not shown).

We then asked whether CsA derivatives like [MeSar]^3 CsA, in which the β-II′ turn was partially lost upon Sar^3 α-C substitution, could mimic the conformation of CsA in the Cyp18–CsA binary complex and inhibit CaN on its own. For this purpose, we measured the Cyp18 inhibition[2,18] as well as the CaN inhibition[19] in the absence or presence of Cyp18 (Table 1). The IC_{50} value of Cyp18 inhibition by [MeSar]^3 CsA is similar to that by CsA. This indicates that a β-II′ turn in CsA is not essential for specific recognition by Cyp18. Cyp18–[MeSar]^3 CsA and Cyp18–CsA complexes also exhibited a similar CaN inhibition behaviour. Interestingly, in the absence of Cyp18, [MeSar]^3 CsA inhibited CaN phosphatase activity with an IC_{50} value of 10 μM. Among the compounds we examined in this study, another

Table 1. *Inhibition of Cyp18 PPIase activity and inhibition of CaN phosphatase activity in the presence or absence of Cyp18 by CsA and its derivatives.*

	Abbreviation	Name of derivatives	IC_{50} of Cyp18 inhibition[d]	IC_{50} of CaN inhibition in the presence of Cyp18[e]	IC_{50} of CaN inhibition in the absence of Cyp18[e]
1	CsA		3.7 nM	100 nM	NI[c]
2	CsH	[D-MeVal]^{11} CsA	NI[a]	NI[b]	NI[c]
3	[MeSar]^3 CsA	[(*R*)α-methyl Sar]^3 CsA	4.0 nM	500 nM	10 μM
4	[DMeSar]^3 CsA	[(*S*)α-methyl Sar]^3 CsA	8.0 nM	40% at 1 μM	NI[c]
5	[MsSar]^3 CsA	[(*R*)α-methylthio Sar]^3 CsA	3.8 nM	1.5 μM	10 μM
6	[DMs Sar]^3 CsA	[(*S*)α-methylthio Sar]^3 CsA	80 nM	NI[b]	NI[c]
7	[MeSar]^3 CsH	[(*R*)α-methyl Sar]^3 CsH	600.0 nM	350 nM	NI[c]
8	[DMeSar]^3 CsH	[(*S*)α-methyl Sar]^3 CsH	NI[a]	NI[b]	NI[c]
9	[AcMeBmt]^1[MeSar]^3 CsA	[acetyl MeBmt]^1 [(*R*)α-methyl Sar]^3 CsA	NI[a]	NI[b]	NI[c]

[a] No inhibition at 1 μM of CsA derivatives; [b] No inhibition at 5 μM of Cyp18; [c] No inhibition at 10 μM of CsA derivatives; [d] SD < 20%; [e] SD < 10%.

compound, that is, [Ms Sar]^3 CsA, exhibited the same effect. This is the first report that CsA derivatives could inhibit CaN in the absence of Cyp18. Because the Cyp18–CsA–CaN complex is further stabilized through direct contacts between Cyp18 and CaN,[20,21] in addition to that between CaN and CsA, it is reasonable that the ligands alone show lower affinity than the Cyp18–CsA complex, which inhibits CaN with an IC_{50} of 100 nM. The biological effects of several key derivatives with modified Sar^3 were reported elsewhere.[22] Functionally, there is an overlap of the Sar^3-modified CsA and the FKBP12–FK506/Cyp18–CsA interacting sites on CaN.

Although [Me Sar]^3 CsA and [Ms Sar]^3 CsA inhibit CaN without forming binary complexes with Cyp18, they do not belong to the monospecific inhibitors because of their high Cyp18 inhibitory potency. The design of a CsA derivative that selectively inhibits CaN without binding to Cyp18 was therefore desirable. For this purpose, CsA analogues were designed and synthesized with a synergism between modifications at the Cyp18 binding domain of CsA and Sar^3 α-C substitution. We assumed that such a combination might lead to both low Cyp18 binding affinity and direct CaN inhibition. Either residue 1 or 11 of CsA was chosen for modification because both positions were found to be essential in Cyp18–CsA interactions.[23] [*O*-Acetyl MeBmt]^1 CsA and [D-MeVal]^{11} CsA (CsH)[24] are more than 2000-fold less active than CsA for Cyp18 inhibition.

As shown in Table 1, although either [Me Sar]^3 CsH or [AcMeBmt]^1[Me Sar]^3 CsA exhibited weak Cyp18 inhibition, as compared to CsA and [Me Sar]^3 CsA, they were not able to act as direct CaN inhibitors. Evidently, additional changes on residue 1 or 11 could have an influence on the entire structure[25] of [Me Sar]^3 CsA, abolishing Cyp18-independent CaN binding properties. On the other hand, [Me Sar]^3 CsH, as compared with CsH, exhibited a remarkable increase in Cyp18 inhibitory potency as well as CaN inhibition by Cyp18–[Me Sar]^3 CsH. CsH is a natural cyclosporin with a D-MeVal11, instead of the L-MeVal11 in CsA. The different orientation of the side chain in the D configuration causes the low affinity of CsH to Cyp18 (Table 1). Although Sar^3 does not appear essential for the binding of CsA to Cyp18,[20,21] its conformational influence could account for the switching of CsH to a CsA-interacting molecule.

In summary, as a proof of concept, we demonstrated that Sar^3 substitutions can influence CsA structure and result in direct CaN inhibition. While the final goal would be to design CaN monospecific CsA derivatives, our current study represents an important step forward, illustrating the structural requirements of CsA analogues for Cyp18-independent CaN binding. Future studies will focus on modifications of [Me Sar]^3 CsA that do not affect Cyp18-independent CaN inhibition, but diminish its Cyp18 active-site affinity.

Experimental Section

Chemicals were bought from Fluka (Germany) and of the best available quality. CD spectra were measured on a J-710 (Jasco) CD-spectrometer. The fluorescence was recorded on a FluoroMax2 (ISA). Modifications of CsA on Sar^3 were carried out according to the procedure reported by Seebach et al.[11] Acetylation of [Me Sar]^3 CsA was performed according to Eberle et al.[26] The identities of all products were confirmed with ESI-MS. [Me Sar]^3 CsA, [DMe Sar]^3 CsA, [Ms Sar]^3 CsA, and [DMs Sar]^3 CsA were analyzed by NMR, and the spectra were same as the published data.[11] The purities of all CsA derivatives are higher than 95%, as analyzed with reverse-phase analytic HPLC.

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