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Synthesis and neurotrophic activity of nonimmunosuppressant cyclosporin A derivatives

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Abstract—In order to exploit cyclophilin as a potential target for neurological drug design, we demonstrate in this presentation that several nonimmunosuppressant analogues of cyclosporin A, modified at the various positions in the 'effector' domain, are equipotent nerve growth agents compared to cyclosporin A. Our results suggest that neurotrophic activity of cyclosporin A and its derivatives resides in the binding domain, and binding to cyclophilin and/or inhibiting rotamase activity may be a necessity for neurotrophic effects of cyclophilin ligands.

The term immunophilin refers to a number of proteins that serve as receptors for the principal immunosuppressant drugs, cyclosporin A (CsA), FK506 and rapamycin.[1,2] Known classes of immunophilins are cyclophilins (CyP), and FK506 binding proteins (FKBP). Cyclosporin A binds to cyclophilin while FK506 and rapamycin bind to FKBP. Immunophilins are also known to possess peptidyl-prolyl isomerase (PPIase) or rotamase enzyme activity, which catalyzes the interconversion of the *cis* and *trans* rotamers of amide bonds adjacent to proline residues in peptidic substrates.[3] Immunophilins were originally discovered and characterized in immune tissue. It was shown that the inhibition of cyclophilin rotamase activity by cyclosporin A, in and of itself, is not sufficient for immunosuppressant activity. Instead immunosuppression appears to stem from the formation of CsA-CyP complexes that inhibit the intrinsic target protein—calcineurin, a calcium/calmodulin dependent protein phosphatase.[4]

Recent findings that cyclophilin A is present at high levels in the CNS and that cyclosporin A possesses neurotrophic effects suggest a potential therapeutic utility for cyclophilin ligands in treating neurological disorders.[5] However, at levels where cyclosporin A exhibits neurotrophic activity, the known immunosuppressant activity of cyclosporin A becomes an unwanted side effect. Therefore, in order to exploit cyclophilin as potential target for neurological drug design, the critical question whether the neurotrophic effect of cyclophilin ligands is separable from the immunosuppressive activity needs to be answered. We have shown previously that a nonimmunosuppressant analogue of cyclosporin A (6-MeVal-CsA) promotes neurite outgrowth in neuronal cultures with potency resembling CsA.[6] In this report, we wish to extend and further validate the previous findings with several additional nonimmunosuppressant cyclosporin A analogues, which are modified at various positions and with various amino acid residues (Fig. 1).

Cyclosporin A is a cyclic undecapeptide, and it possesses two distinct binding domains: a cyclophilin-binding domain, which expends about five amino acids

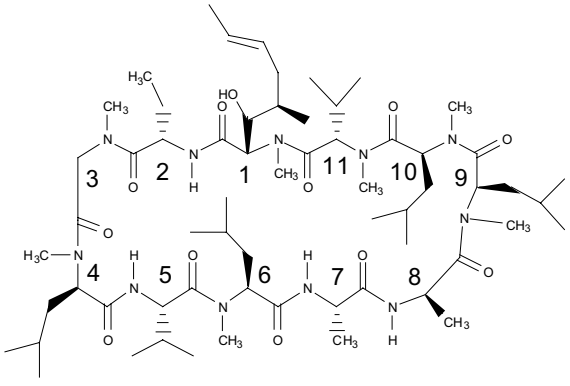

Figure 1. Structure of cyclosporin A.

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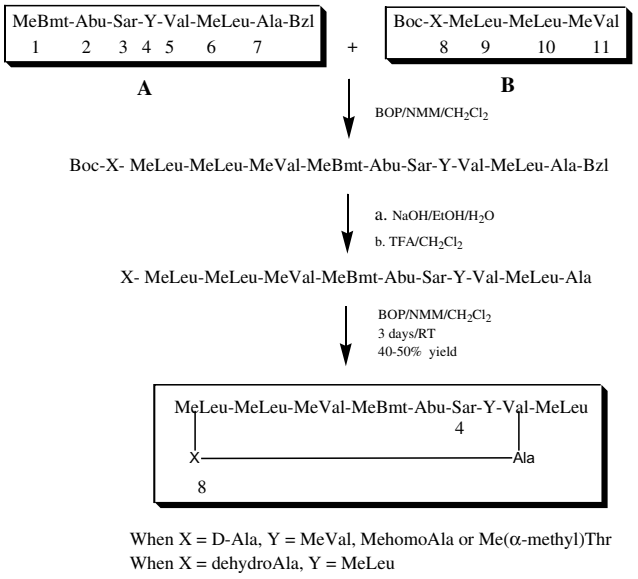
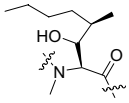
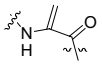
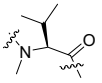
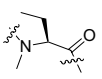
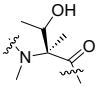


Scheme 1. Total synthesis of cyclosporin A analogues.

(MeLeu9, MeLeu10, MeVal1, MeBmt1 and Abu2), and an 'effector domain', which interacts with calcineurin after the formation of the CsA-CyP complex (Sar3, MeLeu4, Val5, MeLeu6, Ala7). D-Ala8 residue of CsA contacts with neither cyclophilin nor calcineurin directly, suggested by recently solved X-ray crystal structure of CyP-CsA-CN complex.[7] It appears that there is an open, presumably water filled cavity around residue 8 in the ternary complex. It has been known that modification in the 'effector domain' would interrupt activity towards calcineurin, resulting in nonimmunosuppressive compounds. As shown in Scheme 1, we synthesized several cyclosporin A analogues modified at both 4 and 8 positions employing a solution-phase fragment coupling strategy, which was originally developed by Wenger in the first synthesis of CsA.[8,9] Heptapeptide fragments A were constructed by coupling MeBmt residue as an acetonide derivative to hexapeptide precursors, followed by acid deprotection. Fragments A, with various amino acids at position 4, correspond to the residues 1–7 of CsA. Fragments B, which were also synthesized stepwise, correspond to the residues 8–11 of CsA. Position 8 contains either D-Ala or dehydroAla residue. The free amines of fragments A were then coupled to Boc-protected tetrapeptide fragments B, leading to desired linear undecapeptides. Double deprotection of both C-terminals and N-terminals of undecapeptides, followed by cyclization with BOP in dilution condition (<0.25 mM concentration) gave four CsA analogues with yields between 40% and 50%. Separately, dihydroCsA was obtained from CsA by 10% Pd/C catalyzed hydrogenation of double bond at position 1. Synthesized analogues were purified by HPLC, and characterized by ^{1}H NMR and mass spectroscopy, and most of the analogues (Table 1) were reported and known in the literature.[10a,b,c]

The synthesized cyclosporin A analogues were evaluated for cyclophilin A rotamase inhibition by a method previously described.[11] This is a chymotrypsin-coupled assay using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as a substrate. The measured inhibition constants k_i are the mean of at least three independent estimations with variation generally less than 20% (Table 1). Modification at position 4 resulted in several analogues, which are inhibitors for rotamase as potent as cyclosporin A, essentially independent of different amino acid substitutions. However, analogue modified at position 8, which is closer to the binding domain, has threefold less potency than that of CsA against rotamase. Not surprisingly, the saturated dihydroCsA, modified at binding domain, is fivefold less potent than CsA. On the other hand, the analogues modified at position 4 lose immunosuppressive activity (>10 μM) in a standard T-cell proliferation inhibition assay,[12] shown in Table 1.

Table 1. Biological activity of cyclosporin A analogues

Compounds	Modified residue	k_i (nM)/CyP	ED50 (nM) T-cell prolif.	Neutrite outgrowth
CsA[10a]	—	20	50	+++
DihydroCsA[10a]		100	130	+
[DehydroAla]8-CsA[10b]		75	>10,000	+
[MeVal]4-CsA[10c]		10	>10,000	+++
[MeAbu]4-CsA[10c]		24	>10,000	+++
[Me(α-methyl)Thr]4-CsA		18	>10,000	+++

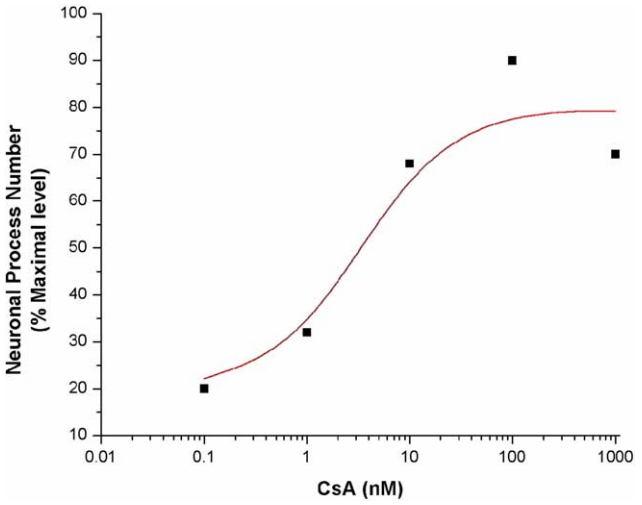


Figure 2. Dose–response curve of cyclosporin A in promoting neurite outgrowth.

Although there are published examples of other D-amino acid substituents at position 8 that retain immunosuppressive activity,[13] dehydroAla8 CsA analogue failed to inhibit T-cell proliferation up to 10 μM, suggesting maintaining a D-configuration at this position may be important for the inhibition of calcineurin activity.

To demonstrate their potential neurotrophic effects, cyclosporin A and its analogues were evaluated in a neurite outgrowth assay using chick dorsal root ganglia (DRG).[6,14] Neurite outgrowth was quantified by the photomicrographs of explants. It was observed that the maximal increase in the number of processes, their length and branching is quite similar at maximally effective concentrations of cyclosporin A and NGF (100 ng/mL). Shown in Figure 2, the ED_{50} of cyclosporin A, the dose at which 50% of the maximal response was elicited, was obtained from dose–response curves, and calculated to be 5 nM. The potencies of the other analogues at concentration of 100 nM (Table 1) are given the relative number of '+' marks denoting, when compared to cyclosporin A.

The data demonstrate that cyclosporin A and its derivatives, which bind to cyclophilin and inhibit rotamase activity, whether immunosuppressive or nonimmunosuppressive, are capable of promoting neurite outgrowth in cultured neurons, and are capable of achieving maximal effects comparable to nerve growth factor itself. In addition, the neurite outgrowth effect of these derivatives is apparently insensitive towards amino acid residue alterations in the 'effector domain'. The combined data imply that neurotrophic activity of cyclosporin A and its derivatives resides in the binding domain, and binding to cyclophilin and/or inhibiting rotamase activity may be a necessity for neurotrophic effects of cyclophilin ligands. At this point, the molecular mechanism of such effects remains elusive, although several possibilities are worth mentioning. One possibility is that interaction of the compounds with cyclophilin or a cyclophilin-like protein results in formation of an active complex, leading to a gain of function for the cyclophilin. Another possibility is that other cyclophilins (e.g., cyclophilin D in mitochondrial), present in lower concentrations in nerve cells, mediate the actions of these compounds.[15] Despite many unanswered questions, cyclophilin may well, in our opinion, serve as an attractive target for small-molecule intervention against neuro-degenerative disorders such as Alzheimer's disease and Parkinson's disease.

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