Design And Synthesis of a Novel Potent Myelin Basic Protein Epitope 87−99 Cyclic Analogue: Enhanced Stability and Biological Properties of Mimics Render Them a Potentially New Class of Immunomodulators†

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A cyclic analogue, [cyclo(87−99)MBP_{87-99}], of the human immunodominant MBP_{87-99} epitope, was designed based on ROESY/NMR distance information and modeling data for linear epitope 87−99, taking into account T-cell (Phe^{89}, Lys^{91}, Pro^{96}) and HLA (His^{88}, Phe^{90}, Ile^{93}) contact side-chain information. The cyclic analogue was found to induce experimental allergic encephalomyelitis (EAE), to bind HLA-DR4, and to increase CD4 T-cell line proliferation, like that of the conformationally related linear MBP_{87-99} epitope peptide. The mutant cyclic peptides, the cyclo(91−99)[Ala^{96}]MBP_{87-99} and the cyclo(87−99)[$Arg^{91}Ala^{96}$]MBP_{87-99}, reported previously for suppressing, to a varying degree, autoimmune encephalomyelitis in a rat animal model, were found in this study to possess the following immunomodulatory properties: (i) they suppressed the proliferation of a CD4 T-cell line raised from a multiple sclerosis patient, (ii) they scored the best in vitro TH2/TH1 cytokine ratio in peripheral blood mononuclear cell cultures derived from 13 multiple sclerosis patients, inducing IL-10 selectively, and (iii) they bound to HLA-DR4, first to be reported for cyclic MBP peptides. In addition, cyclic peptides were found to be more stable to lysosomal enzymes and Cathepsin B, D, and H, compared to their linear counterparts. Taken together, these data render cyclic mimics as putative drugs for treating multiple sclerosis and potentially other Th1-mediated autoimmune diseases.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by discrete areas of inflammation and demyelination that can occur in multiple anatomical locations in the CNS.[1−5] $CD4^+$ T helper cells reactive to myelin, which produce proinflammatory cytokines, such as interferon-γ (IFN-γ), are known to have a leading role in the pathogenesis of MS.[6] Myelin basic protein (MBP) is a major autoantigen defined in MS, and in rodents it induces an MS-like disease, experimental autoimmune encephalomyelitis (EAE). In rats, EAE is a TH1 $CD4^+$ T-cell-mediated disease and is induced by immunization with MBP proteins or peptides.[7] Due to the adverse effects and marginal benefits of current treatments available for MS, such as interferons (IFNs) and the copolymer glatiramer acetate (derived from the major amino acids Glu, Ala, Lys, Tyr of MBP), new therapeutic approaches are sought.[8] Such an approach, involves the design and use of peptide analogues of disease-associated myelin epitopes to modify T-cell responses.[9] Altered peptide ligands (APL), which are analogues of MBP epitopes (i.e. MBP_{1-11} MBP_{87-99}, MBP_{84-102}, MBP_{87-106}, and MBP_{83-99}), inhibit the development of EAE induced by the parent peptides.[10−12] Antagonistic effect (i.e., loss of T-cell activation) is due to the loss of H-bond contacts between the peptide and the T-cell receptor (TCR). In addition, APL can switch TH1 (IFNγ) immune response toward TH2 (IL-10).[13] A linear APL of the immunodominant epitope MBP_{83-99} was injected into patients with MS in a phase I clinical trial; however, it was suspended due to adverse side effects.[5]

We are investigating MBP_{87-99} cyclic analogues, as possible immunomodulators in MS treatment, because they are more stable and selective, compared to their linear counterparts. Our synthetic approaches focus toward constrained cyclic analogues which will inhibit the formation of the MHC-peptide−T-cell receptor trimolecular complex, implicated in the pathogenesis of the disease, and/or have immunomodulatory activity that

† This article is dedicated to the late Marco Vergelli.

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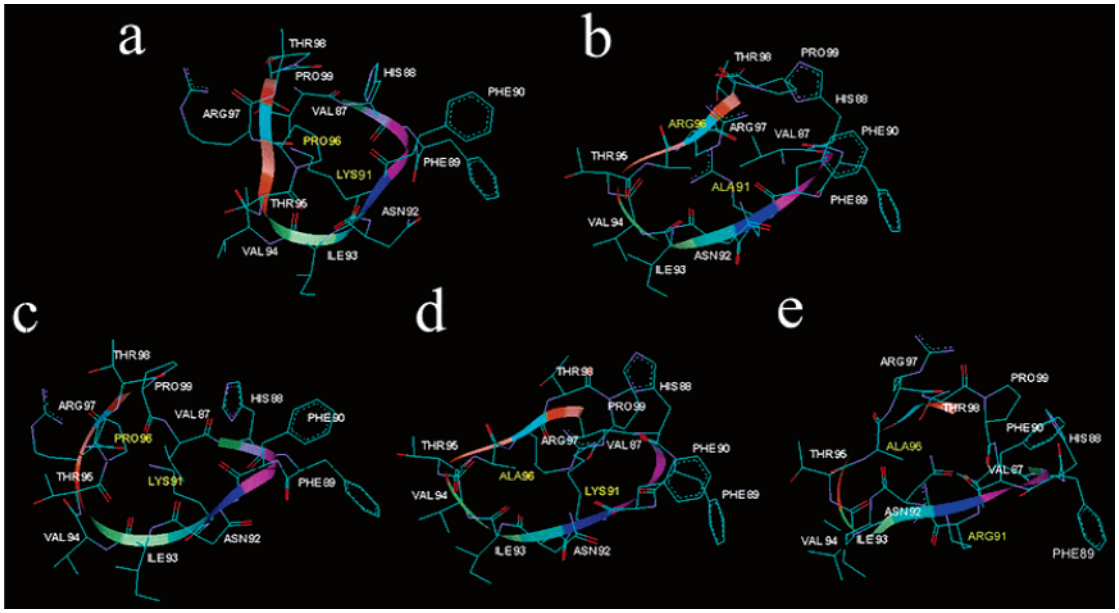


Figure 1. Low energy conformers of MBP_{87-99} peptide analogues. a, linear agonist MBP_{87-99} (P1); b, linear antagonist [$Arg^{91}Ala^{96}$]MBP_{87-99} (P2); c, cyclic agonist cyclo(87−99)MBP_{87-99} (P3); d, cyclic antagonist, cyclo(91−99)[Ala^{96}]MBP_{87-99} (P4) and e, cyclic antagonist, cyclo(87−99)[$Arg^{91}Ala^{96}$]MBP_{87-99} (P5).

prevents or controls the disease though the release of appropriate cytokines.

The present study describes the design and synthesis of a novel head-to-tail cyclic analogue (**P3**) (Figure 1) of the immunodominant epitope sequence MBP_{87-99} and pharmacological properties of mutant peptides thereof that have amino acid changes at positions 91 and 96 identified as TCR contact sites.[14] The rational design and synthesis of these peptides was based on a combination of conformational analysis studies and theoretical calculations carried out on the linear MBP epitope 87−99 (**P1**) using 2D NMR spectroscopy.[15] These studies have revealed a head-to-tail intramolecular proximity between Val^{87}-Arg^{97} residues, suggesting a near cyclic conformation for the linear peptide.[15] In particular, NOE connectivities between distant residues, γ-Val^{87}-NH Arg^{97} and ϵ-NH-Lys^{91}-$\omega/\acute{\omega}$-Arg^{97} for linear epitope 87−99 suggested a near cyclic conformation, thus directing us to the design and synthesize cyclic analogue **P3**. The low energy conformers of MBP_{87-99} peptide analogues **P2**−**P5** were derived from template **P1** using mutation amino acid structural changes (see Experimental Procedures). In this respect, the linear antagonist **P2**, as well as cyclic **P3**−**P5**, retained similar conformation. Energy minimization using the steepest descent algorithm was performed to obtain an energy gradient tolerance of 0.01 kcal·mol^{-1} for compounds **P2**−**P5**. NMR soectroscopy is under progress using a 600 MHz spectrometer, in which the final conformational analysis of **P1**−**P5** will be sought based on quantitative NOE data.

Cyclization provides stability, since the use of linear peptides as therapeutic entities, in general, is limited because of their sensitivity to proteolytic enzymes. Therefore, to address the need for a more stable molecule for clinical purposes, it is necessary to use either cyclic peptides that are more resistant to proteolytic hydrolysis or nonpeptide mimetics of the parent peptide. While mimetic strategy is a challenging perspective, it is worth pursuing in particular for MBP epitope-based MS therapy, as it is still in its infancy. Efforts to design semimimetics of MBP_{72-85} epitope (the guinea pig epitope) by combining nonnatural amino acids (iNip, Acp) as spacers and MBP epitope immunophores (Ser, Arg, Glu, Ala, Gln) led to substances which were effective to some extent in inducing the onset of EAE.[16−18] Presently, our studies focus mainly on the design, synthesis, and evaluation of cyclic mimics not only as a step toward nonpeptide mimetics but also as putative therapeutics in MS. The advantages of using cyclic analogues compared to linear peptides include the following: (i) cyclic analogues are more stable and more resistant to enzymatic degradation; (ii) cyclization of amino acid sequences results in increased receptor selectivity, consequently resulting in an improved pharmacological profile; (iii) the conformation of cyclic analogues is locked compared to the conformational flexibility characterizing the linear counterpart, allowing identification of active sites; (iv) cyclic analogues are an important intermediate step and a useful template toward the rational design and development of a nonpeptide (mimetic) drug for oral administration; and (v) it is feasible that a cyclic peptide could be orally active if appropriately constrained. Bioassay results in the EAE animal model have shown that cyclic analogues have comparable effects with the linear peptides. In particular, we had previously demonstrated that injection of Lewis rats with linear agonist guinea pig MBP_{72-85} peptide (QKSQRSQDENPV) induced EAE; cyclization of MBP_{72-85} also induced EAE.[16,19] Alteration of one amino acid at position 81 from this epitope, i.e., aspartic acid to alanine (QKSQRSQAENPV), resulted in the antagonist [Ala^{81}]MBP_{72-85} which inhibited EAE when coinjected with the linear or cyclic agonist peptide.[19,20] In addition, human MBP_{87-99} (VHFFKNIVTPRTP) linear epitope induced EAE in rats, which was inhibited by the antagonist linear peptide [Arg^{91}, Ala^{96}]MBP_{87-99} (VHFFRNIVTARTP) or antagonist cyclic peptide, cyclo (87−99) [Arg^{91} Ala^{96}]MBP_{87-99}. Furthermore, coinjection of linear guinea pig MBP_{72-85}

Table 1. ^{1}H NMR Assignments, Chemical Shifts (δ, ppm) and NH Temperature Coefficients ($-\Delta\delta/\Delta T$) of Agonist MBP_{87-99} and Antagonist [Arg^{91},Ala^{96}]MBP_{87-99} in DMSO-d_6 at 300 K

amino acid	NH	α	β	other	-$\Delta\delta/\Delta$T (ppb/K)
			Agonist MBP_{87-99} (P1)		
Val 1	-	3.58	1.96	γ: 0.83	-
His 2	8.60	4.64	2.96, 2.89	2H: 8.92, 4H: 7.22	3.88
Phe 3	8.20	4.56	3.00, 2.75	ring: 7.29−7.11	4.11
Phe 4	8.34	4.58	3.05, 2.84	ring: 7.29−7.11	5.01
Lys 5	8.13	4.33	1.64	γ: 1.31, δ: 1.53, ϵ: 2.75, ζ: 7.70	5.82
Asn 6	8.27	4.61	2.53, 2.41	NH_2: 7.39, 6.95	5.39
Ile 7	7.74	4.23	1.74	γ_1: 1.38, γ_2: 1.07, δ: 0.81	3.42
Val 8	7.81	4.23	1.96	γ: 0.83, 0.79	3.66
Thr 9	7.93	4.39	3.85	γ: 1.12	7.33
Pro 10	-	4.33	2.13, 1.91	γ: 1.87, δ: 3.77, 3.61	-
Arg 11	8.00	4.30	1.68	γ: 1.48, δ: 3.08, ϵ: 7.46	4.17
Thr 12	7.88	4.32	3.82	γ: 1.14	6.40
Pro 13	-	4.20	2.15, 1.91	γ: 1.84, δ: 3.78, 3.64	-
			Antagonist [Arg^{91}, Ala^{96}]MBP_{87-99} (P2)		
Val 1	-	3.59	1.96	γ: 0.82	-
His 2	8.60	4.65	2.95, 2.90	2H: 8.92, 4H: 7.23	3.43
Phe 3	8.19	4.55	3.00, 2.75	ring: 7.25−7.13	3.85
Phe 4	8.34	4.60	3.05, 2.84	ring: 7.25−7.13	5.21
Arg 5	8.20	4.36	1.70	γ: 1.50, δ: 3.10, ϵ: 7.60	5.54
Asn 6	8.25	4.62	2.54, 2.42	NH2: 7.40, 6.96	4.70
Ile 7	7.76	4.21	1.72	γ_1: 1.38, γ_2: 1.04, δ: 0.79	3.91
Val 8	7.90	4.19	1.96	γ: 0.85	4.77
Thr 9	7.78	4.22	4.01	γ: 1.01	3.71
Ala 10	7.83	4.28	1.22		2.47
Arg 11	7.98	4.32	1.66	γ: 1.47, δ: 3.07, ϵ: 7.51	3.82
Thr 12	7.90	4.32	3.81	γ: 1.14, OH: 4.91	6.57
Pro 13	-	4.19	2.14	γ: 1.83, δ: 3.79, 3.64	-

agonist peptide with linear or cyclic human [Arg^{91}, Ala^{96}]MBP_{87-99} antagonist peptide inhibited EAE in Lewis rats.[15,21]

Herein, we demonstrate that rationally designed cyclic peptide **P3**, based on human MBP_{87-99}, induces EAE in rats, binds to HLA-DR4, which is the first to be reported for cyclic MBP peptide mimics, and increases CD4 T-cell line proliferation, like that of the conformationally related linear MBP_{87-99} epitope peptide. We furthermore demonstrate that **P3** mutant cyclic mimics **P4** and **P5** suppress CD4 T-cell line proliferation, in line with their antagonist effects in EAE with **P4**, to score the best TH2/TH1 cytokine ratio with MS patients' peripheral blood mononuclear cells (PBMC), rendering EAE antagonists potential therapeutic agents for treating disease in humans. Cyclic analogues **P3**−**P5** were found to be more stable to lysozomal enzymes compared to linear peptides.

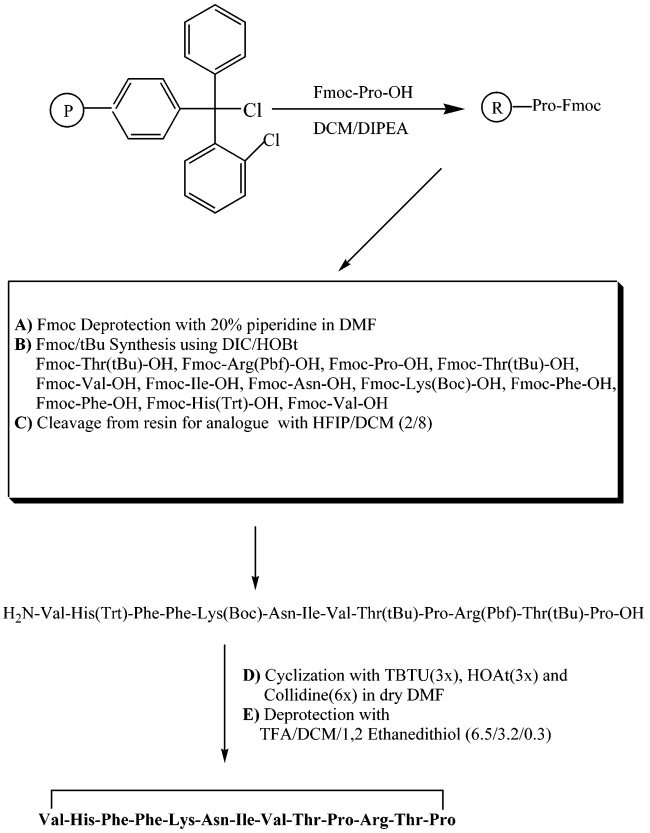
Results

Assignment and Conformational Analysis. Two-dimensional total correlated spectroscopy (TOCSY) was used allowing residue identification and the assignment of all protons, while Overhauser spectroscopy (ROESY) technique was applied in order for ROE connectivity patterns to be established for distance information in linear agonist (**P1**) and antagonist (**P2**) peptides. There were almost no overlapping cross-peaks in the $H^\alpha-H^N$ region of the two-dimensional TOCSY experiments of the two peptides. Using row or column projections for all the cross-peaks in the $H^\alpha-H^N$ region, the residue identification and the complete assignment of all protons for both peptides were obtained. The proline residues were identified by their δ-protons, in a manner similar to that previously described.[32a,b]

In particular, examining the ROESY spectrum of the agonist, the following medium $H^N(i)-H^N(i+1)$ cross-peaks were observed: His^2-Phe^3, Phe^3-Phe^4, Phe^4-Lys^5, Lys^5-Asn^6, Asn^6-Ile^7, Val^8-Thr^9, and Arg^{11}-Thr^{12}, indicating a backbone turn in the His^2-Ile^7 segment. Also, a weak Ile^7-Thr^{12} cross-peak was seen, indicating a bend in the C-terminal segment. Strong $H^\alpha(i)-H^N(i+1)$ cross-peaks were observed in all cases, furthermore supporting a backbone turn. Apparently, such cross-peak was not observed, when a proline residue was present in the i+1 position. The existence of these ROEs led us to look for a weak $H^\alpha(i)-H^N(i+2)$ or a weak $H^\beta(i)-H^N(i+2)$ cross-peak, to identify a type II β-turn. No such cross-peak was found in our spectrum. Since, the existence of consecutive $H^N(i)-H^N(i+1)$ cross-peaks is an indication for the presence of a turn, we can assume that a backbone turn (probably a U-type turn) exists in the segment Val^1-His^2-Phe^3-Phe^4-Lys^5-Asn^6-Ile^7, which is also confirmed by a weak Val^1 $H^\alpha-Lys^5$ H^N cross-peak. A second turn is expected in the segment Val^8-Thr^9-Pro^{10}-Arg^{11}-Thr^{12}-Pro^{13}, due to the existence of the weak Ile^7 H^N-Thr^{12} H^N cross-peak. Examining the ROESY spectrum of the antagonist, the medium Ala^{10} H^N-Arg^{11} H^N and the strong Thr^9 $H^\alpha-Ala^{10}$ H^N cross-peaks were observed, besides the other $H^N(i)-H^N(i+1)$ and $H^\alpha(i)-H^N(i+1)$ cross-peaks observed also in the agonist peptide. In addition, almost the same number of interresidue NOE connectivities was observed for both peptides. These results, together with the almost identical proton chemical shifts which were observed for the protons of the common amino acid residues in both epitopes, indicate a likewise conformation for the two peptides (Table 1).

Novel Cyclization Method. Scheme 1 shows the synthesis of novel head-to-tail cyclic peptide agonist, cyclo(87−99)MBP_{87-99} (**P3**). For the synthesis of the cyclic peptide **P3**, acid-sensitive 2-chlorotrityl chloride resin (CLTR-Cl), appropriate side chain protection, and

Scheme 1. Synthetic Procedure for Cyclic Analogue: Cyclo(87−99)MBP_{87-99} (P3)



Fmoc/tBu methodology were used and cyclization was achieved using TBTU/HOAt and collidine as base.[15,19,20] These reagents induced fast, quantitative reactions without side products. In particular the side chains of amino acids were protected with Trt for His, Pbf for Arg, tBu for Ser, Thr, Asp, Glu, and Boc for Lys. The use of the 2-chlorotrityl resin, as well as of mild cleaving conditions, allowed peptide release from the resin with side chain protection (except for the termini NH_2 and COOH groups) and the subsequent cyclization of the desired protected peptide. Cyclization was achieved using *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxy-7-azabenzotriazole, and 2,4,6 collidine, allowing fast reaction and high yield cyclization product. The cyclization reaction was monitored using the ninhydrin test and analytical HPLC. The reaction mixture was resolved by thin-layer chromatography using an *n*-butanol/acetic acid/water (4/1/1) solvent system. The protected cyclic analogue was then deprotected with 65% trifluoroacetic acid (TFA) + 3% EDT in DCM. The final linear and cyclic peptides were purified by reverse phase HPLC using a Lichrosorb RP-18 semipreparative column and analyzed by mass spectrometry and analytical RP-HPLC. Peptides were >98% pure. Peptide structures were confirmed by electron spray ionization mass spectrometry. This strategy is superior compared to other methods using Merrifield type resins and cyclization agents such as HBTU, HOBt, and DIPEA, as the acid-sensitive CLTR-Cl resin[22] which we used allows for selective deprotection.[15,19−21,23−28]

Induction of EAE with Cyclic MBP_{87-99} (P3). We have previously demonstrated that the linear human MBP_{87-99} peptide induces weak encephalitogenic activity in Lewis rats.[15] Similarly, the cyclic MBP_{87-99} analogue **P3** induced weak EAE (clinical score 1). The use of human MBP_{87-99} agonist peptides in Lewis rats results in anaphylactic shock and weak signs of EAE. On the contrary, guinea pig (gp) epitope MBP_{72-85} agonist peptide induced strong signs of EAE in Lewis rats. Therefore, human antagonist peptides **P2**, **P4**, **P5**, were mainly screened in assays in which EAE was induced by $gpMBP_{72-85}$. Clinical signs of EAE induced by MBP_{87-99} or by MBP_{72-85} were completely suppressed by head-to-tail cyclic analogue **P5** and to a lesser degree by the Lys^{91} side chain to the C-terminus cyclic analogue **P4**.[15] Overall, the cyclic peptides **P4** and **P5** were able to suppress the development of EAE when coinjected with MBP_{72-85} or MBP_{87-99} peptides.

Effect of Cyclic Peptides on a CD4+T-Cell Line Derived from an MS Patient. The in vitro generated CD4+T-cell line (specific for MBP_{80-99}, **P1**) was tested for proliferative activity to **P1**−**P5** peptides. The linear MBP_{87-99} peptide (**P1**) and cyclo(87−99)MBP_{87-99} (**P3**) induced T-cell proliferation. In contrast, the antagonist cyclic peptides, cyclo(91−96)[Ala^{96}]MBP_{87-99} (**P4**) and cyclo(87−99)[Arg^{91}, Ala^{96}]MBP_{87-99} (**P5**), inhibited proliferation of the T-cell line as did the linear antagonist [Arg^{91}, Ala^{96}]MBP_{87-99} (**P2**) (Figure 2).

Effect of Peptides on Th1 and Th2 Cytokine Secretion by MS Peripheral Blood Mononuclear Cells Cultures from MS Patients and Controls. To determine the cytokine secretion pattern, an in vitro ELISA assay measuring cytokines was used. Cells from healthy controls and MS patients were cultured for 72 h with or without **P1**−**P5** peptides. Supernatants were collected, and their cytokine content was measured by ELISA. The values obtained for all cytokines tested in the control PBMC cultures were negligible (not shown). Constitutive cytokine production by MS patients PBMC consisted mainly of IFN-γ and IL-10, while constitutive production of IL-2 and IL-4 was low to negligible (not shown); thus, the ratio Th2/Th1 for the MS patients represents their IL-10/IFN-γ ratio (Figure 3). Th2 cytokine production by PBMC of healthy controls was increased by 3.5-fold (**P2**) and 15−24-fold (**P1**, **P3**, **P5**) with the exception of **P4** that had no effect on cytokine production by control PBMC. The addition of peptides **P2** and **P4** to PBMC cultures of MS patients significantly increased the Th2/Th1 cytokine ratio in 5 of 12 patients, with the best effect achieved by **P4** (average $\times$2.3-fold), while it moderately decreased the Th2/Th1 cytokine ratio in 7 of 12 patients (average 1.65−1.7-fold, respectively). In contrast, addition of peptides **P1**, **P3**, **P5** to PBMC from MS patients resulted in a decrease of the Th2/Th1 ratio in all the patients, with the worst effect achieved by peptides **P1** and **P3** (average of 4.0−4.7-fold, respectively). Peptides **P2**, **P4** had a beneficial immunosuppressive (high IL-10/low IFN-γ) effect in 42% of human MS PBMC studied and warrant further investigation. Out of the two, peptide **P4** is the most attractive candidate as a drug lead for the following reasons: (i) It has no effect on Th1 or Th2 cytokine production by control PBMC, (ii) it scores the best Th2/Th1 cytokine ratio with MS patients PBMC, and (iii) being cyclic, it is stable for in vivo use.

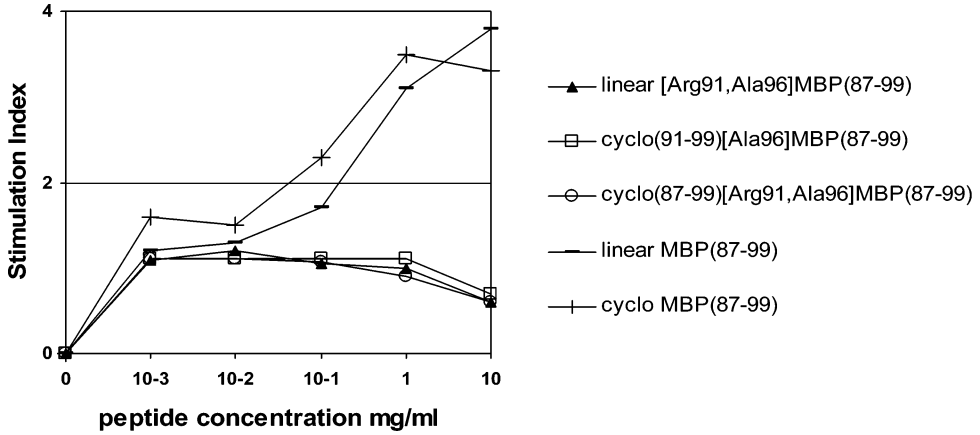


Figure 2. In vitro proliferation of T-cell line from a patient with MS. The T-cell line proliferated only in the presence of linear MBP_{87-99} and cycloMBP_{87-99}. Cyclo(87−99)[Arg^{91},Ala^{96}]MBP_{87-99} and cyclo(91−99)[Ala^{96}]MBP_{87-99} seem to behave as antagonist of the linear MBP_{87-99}, in fact they show an activity similar to the linear antagonist [Arg^{91},Ala^{96}]MBP_{87-99}.

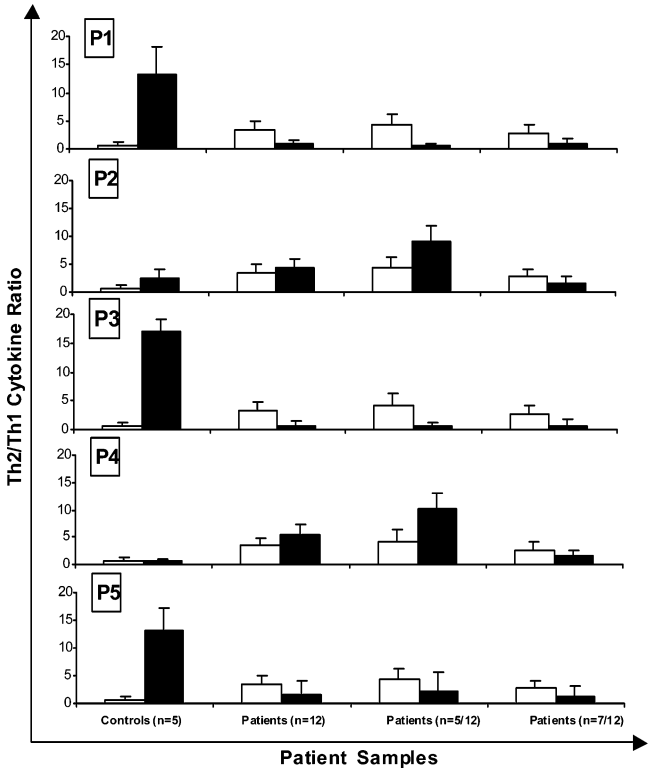


Figure 3. Cytokine production of PBMC from MS or non-MS patients. The Th2/Th1 cytokine ratio is plotted on the *y*-axis and the patient samples on the *x*-axis.

Binding of P1−P5 Peptides to HLA-DR4 (DRB1*0401). To determine the binding ability of cyclic peptides to HLA-DR4, an in vitro competition assay based on gel filtration was used.[29,30] We used fluorescent AMCA-labeled allele-specific $HA_{306-318}$-peptide and immunoaffinity-purified HLA-DR isolates from EBV transformed cells. As competitors, the MBP peptides were used in different concentrations. Guinea pig epitope MBP_{72-85} did not bind to HLA-DR4 (negative control peptide, not shown). Linear peptides (**P1**, **P2**) bound strongly to HLA-DR4, and cyclic peptides (**P3**, **P5**) bound with comparable affinities (Figure 4). **P4**, which forms an internal bridge between 91 and 99, has reduced binding capacity. This is in agreement with the altered conformation which does not allow an optimal accommodation into the binding groove.

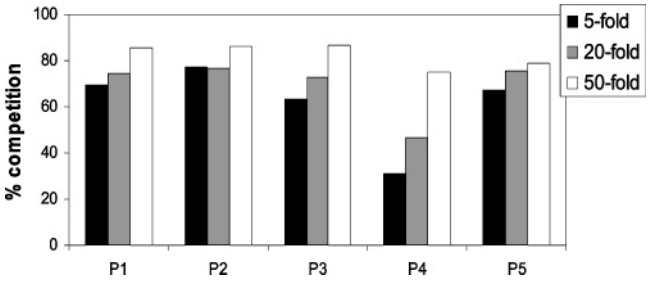


Figure 4. Binding of linear and cyclic MBP peptides (P1−P5) to HLA-DR4. Competition binding at 5-fold (black), 20-fold (grey) and 50-fold excess of MBP peptides. % competition is shown on the *y*-axis and peptides (P1−P5) are labeled on the *x*-axis.

Stability of Cyclic Peptides with Lysosomal Enzymes. To determine the stability of cyclic versus linear MBP_{87-99} peptides (**P1**−**P5**), we used (i) a lysosomal fraction of an EBV-transformed B cell line, (ii) Cathepsin D, and (iii) exopeptidases, Cathepsin B and Cathepsin H. The lysosomal fraction, isolated from the EBV-transformed B cell line BSM, contains all potential lysosomal enzymes (>50 different lysosomal enzymes). The antigen processing and loading compartments of antigen-presenting cells are occupied by a series of cysteine proteinases, in particular Cathepsins B, L, S, H, and AEP and aspartic proteases Cathepsin D and E. The dominant enzymes in this fraction are Cathepsin D and asparaginyl endopeptidase [AEP; a cysteine proteinase, which specifically cleaves after N and is described as an essential enzyme for antigen processing[31]]. The linear peptides **P1** and **P2** were not stable when digested with the lysosomal fraction, whereas the cyclic peptides were clearly stable, **P3** and **P5** being most stable and **P4** being of intermediate stability (Figure 5a). Similarly, Cathepsin D digestion of peptides show an increased stability of the cyclic peptides (**P3**, **P4**, **P5**) compared to the linear ones (**P1**, **P2**) (Figure 5 *b*). Cathepsin D is an aspartic protease which cleaves predominantly between hydrophobic amino acids, and in MBP between Phe_{89}−Phe_{90}.[31] Furthermore, digestion with the exopeptidases − Cathepsin B (peptidyldipeptidase, carboxypeptidase) and Cathepsin H (aminopeptidase) which cleave peptides from the C-terminus, demonstrated that cyclic peptides are clearly more stable than the linear peptides (Figure 5 *c*). It is clear,

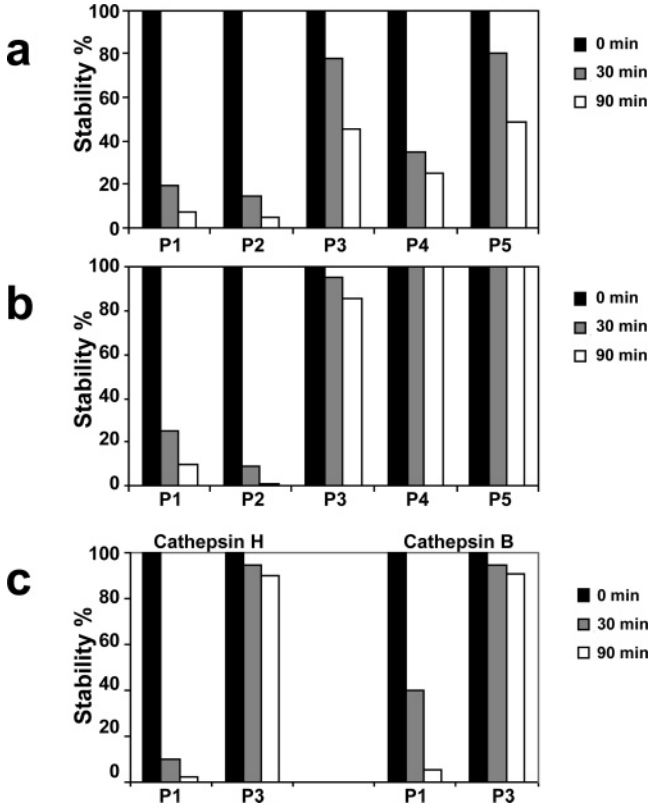


Figure 5. Stability of P1−P5 MBP peptides in the presence of lysozymal enzymes. a, lysozymal fraction; b, lysozymal protease Cathepsin D and c, lysozymal exopeptidase Cathepsin B and Cathepsin H. Stability of the peptides after digestion at different time points (0 min, black; 30 min, gray; 90 min, white) is shown on the *y*-axis. Peptides are labeled P1−P5 on the *x*-axis.

that all enzymatic cleavages show greatly improved stability for the cyclic MBP_{87-99} peptides (**P3**−**P5**) compared to the linear MBP_{87-99} peptides (**P1**, **P2**).

Discussion

In the present study we have synthesized a novel head-to-tail cyclic analogue, cyclo MBP_{87-99} based on ROESY connectivities observed in linear 87−99 MBP epitope. In particular cyclic analogue was found to exhibit pharmacological properties, in a number of assays, similar to that shown by counterpart linear MBP_{87-99} epitope thus justifying NMR based design strategy. In particular, the long-range ROE connectivity between the α-CH proton of Val^1 and the α-NH proton of Arg^{11}, which is observed in agonist's molecule, together with the long-range ROE connectivity between the α-CH proton of Val^1 and the β-CH proton of Thr^{12}, which is observed in antagonist's molecule, indicated a possible head-to-tail interaction in both linear molecules. This conformation was also suggested by a number of observed long-range ROE connectivities, such as the ϵ-NH proton of Arg^{97} and the γ-CH_3 proton of Thr^{95} connectivity with the ring protons of Phe^{89} or Phe^{90}, not possible to be differentiated as overlapping (Figure 6). Molecular modeling based on critical interresidue ROEs (Table 2) identified in our ROESY experiment led to a 3-D model of a linear MBP_{87-99} epitope in which the two terminal residues Val^{87} and Pro^{99} are in close proximity (Figure 7). The calculated HN-CH dihedral angles for the ^3J coupling constants are in very

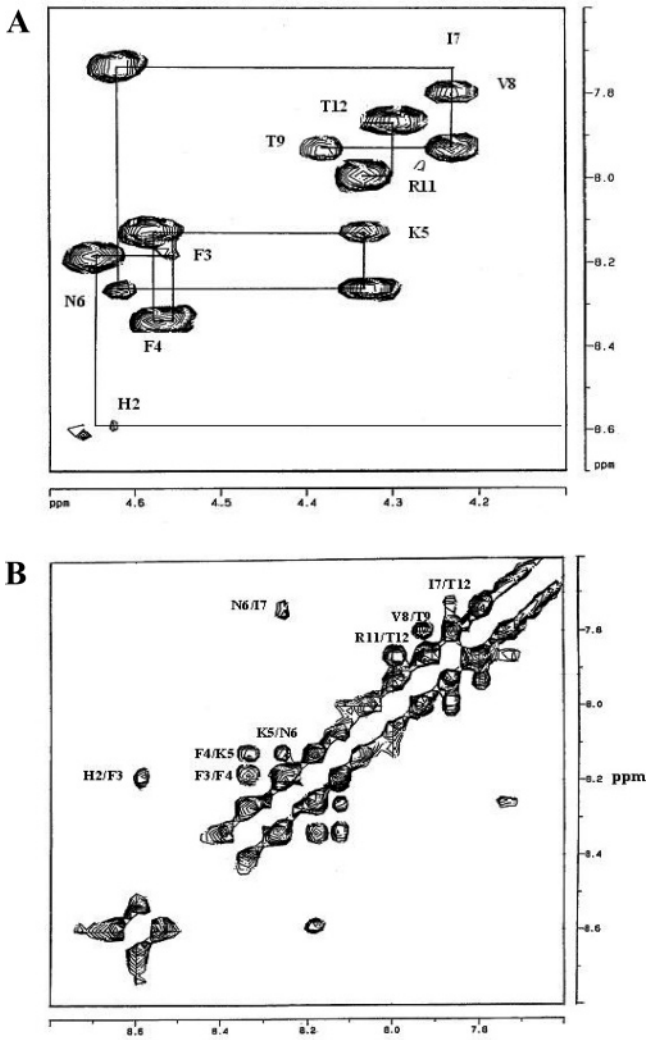


Figure 6. (A) The $H^{\alpha}(\delta_2)-H^{N}(\delta_1)$ region of the ROESY spectrum of the human MBP_{87-99} epitope. Sequential NOE connectivities corresponding to intraresidue $H^{\alpha}(i)-H^{N}(i)$ and sort range interresidue $H^{\alpha}(i)-H^{N}(i+1)$ are shown, but only the intraresidue NOEs are labeled for simplicity. The Val^1 H^{α}-His^2-H^{N} cross-peak is outside of the region. (B) The $H^{N}(\delta_1)-H^{N}(\delta_2)$ region of the same spectrum. A weak cross-peak corresponding to Ile^7 H^{N}-Thr^{12} H^{N} is observed, indicating a bend in the C-terminal segment.

good agreement with those obtained by molecular modeling which is based on inter-residue ROE connectivities (Table 3).

Furthermore, we investigated the effects of linear and cyclic APLs based on the sequence of the immunodominant MBP_{87-99}, both in vivo (EAE model) and in vitro (human PBMC and T-cell line). The importance of the MBP_{83-99} sequence in human studies of MS results from a number of experimental findings. MBP peptides (89−101 and 87−99) are encephalitogenic in rodent strains susceptible to acute (Lewis rat) and chronic (SJL mouse) EAE.[13] Since MBP is one of the candidate autoantigens in MS, and the MBP_{87-99} epitope represents the most immunodominant region in human MS, the design of cyclic analogues with immunomodulatory activity is of great importance. We therefore designed and synthesized the cyclic peptide **P3** which together with peptides **P1**, **P2**, **P4**, **P5** were evaluated for their effects in animal models as well as with human T-cell lines and PBMC. In this regard we have recently reported EAE

Table 2. Interresidue NOE Connectivities for Linear MBP_{87-99} Epitope in DMSO

MBP_{87-99} (agonist)		MBP_{87-99} (agonist)	
NH: His2−NH: Phe3	m	NH: Thr12−ring	w
NH: His2−α: Val1	s	NH: Val8−α: Ile7	s
NH: His2−β: Val1	m	NH: Val8−β: Ile7	w
NH: His2−γ: Val1	w	NH: Val8−NH: Thr9	m
NH: Phe4−NH: Lys5	m	NH: Ile7−α: Asn6	s
NH: Phe4−NH: Phe3	m	NH: Ile7−ring	w
NH: Phe4−α: Phe4	s	NH: Ile7−NH: Thr12	w
NH: Phe4−β_1: Phe3	w	ζNH: Lys5−ring	w
NH: Asn6−α: Lys5	s	ϵNH: Arg11- ring	w
NH: Asn6−NH: Ile7	w	γ_1NH: Asn6−α: Thr9	w
NH: Phe3−α: His2	s	γ_1NH: Asn6−α: Ile7 or Val8	w
NH: Lys5−α: Phe4	s	γ_1NH: Asn6−ring	m
NH: Lys5−α: Val1	w	γ_1NH: Asn6−ω/ωNH: Arg11	w
NH: Arg11−α: Pro10	s	β_2: Asn6−ring	w
NH: Arg11−α: Val1	w	γ_2: Val8−ring	w
NH: Thr9−NH: Val8	m	β_1: Asn6−ring	w
NH: Thr9−α: Val8	s	ϵ: Lys5−ring	m
NH: Thr9−ring	l	γ_1: Val8 −ring	w
NH: Thr9− β: Val8	m	γ_2: Val8−ring	m
NH: Thr9−γ_1: Val8	w	α: Thr9−$\delta_{1,2}$: Pro10	m
NH: Thr12−NH: Arg11	m	α: Pro13−β: Thr12	w
NH: Thr12−α: Arg11	s	δ_2: Pro10−γ: Thr9	m

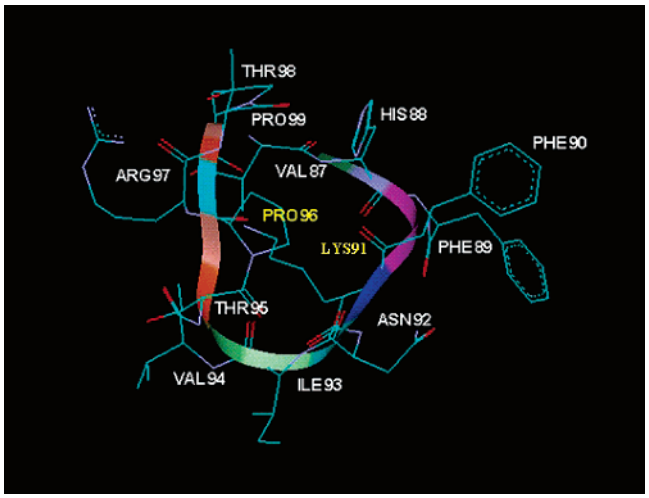


Figure 7. 3-D Model of linear MBP_{87-99} epitope based on interresidue NOE connectivities.

antagonist activities for cyclic analogues cyclo(91−99)-[Ala^{96}]MBP_{87-99} (**P4**) and cyclo-(87−99)[Arg^{91}, Ala^{96}]-MBP_{87-99} (**P5**) designed based on the human MBP antagonist analogue cyclo (87−99) [Arg,[91] Ala^{96}] (MBP_{87-99}) **P2**.[15]

EAE, an experimental animal model of MS, is an inflammatory $CD4^+$ T-cell-mediated disease that can be induced by injection into Lewis rat peptide epitopes from human MBP protein. The epitope identified in guinea pig which induces EAE is MBP_{72-85}. The immunodominant epitope identified in humans is MBP_{85-99} or MBP_{87-99}. Substituting one or two amino acids from the parent peptide at certain positions prevents disease in EAE. Since the peptides that bind to MHC class II molecules have been determined to involve a minimum of 13aa residues which satisfy a particular motif, the design of a minimal size cyclic mimetic that would maintain their functional role in vivo, as well the appropriate side chain substitutions for EAE inhibition, is quite challenging. Structure−activity studies have identified positions 81 in guinea pig MBP_{72-85} and 91, 96 in human MBP_{83-99}, critical for TCR recognition and therefore for inhibition effects. The binding of such APL to the TCR can prevent disease through one of several different mechanisms, including TCR antagonism,[33,34] induction of energy in responding cells, or the stimulation of immunoregulatory T-cells that can actively modulate disease outcome through the secretion of TH2 cytokines. However, the development of alternative molecules that will mimic the immunomodulatory activity of MBP CD4 T-cell epitope peptides and maintain an advantage over regular (linear) peptides in terms of stability is a necessary step before they can be used for therapeutic purposes in animal models or in humans. To address the need for more stable molecules, it was necessary to pursue cyclic analogues of MBP, as they are resistant to proteolysis. Cyclic peptides have been shown to be better vaccine/drugs, as they restrict the number of possible conformations, allowing the possibility to mimic the native structure. In particular, cyclic peptides have been demonstrated to protect mice from diabetes,[35,36] to be potent inhibitors in several models,[37,38] synthetic immunogens,[39] antigens,[40] and protein stabilizers.[41] The design of stable peptides is of great interest, since the limited stability of linear peptides often severely restricts their medical application. Related work in our laboratory has led to the design and synthesis of cyclic analogues for guinea pig MBP_{72-85}, Thrombin receptor motif SFLLR, Angiotensins II and III, and GnRH (unpublished) which were able to maintain or suppress the biological function of the original peptide.[15,20,23−28]

We had previously demonstrated that injection of Lewis rats with linear or cyclic guinea pig MBP_{72-85} peptides induced EAE.[19] Coinjection of linear guinea pig MBP_{72-85} agonist peptide with human cyclic [Arg^{91}, Ala^{96}]MBP_{87-99} antagonist peptide inhibited EAE.[15] In addition, coinjection of linear guinea pig MBP_{72-85} agonist peptide with the cyclic human [Arg^{91}, Ala^{96}]-MBP_{87-99} antagonist peptide inhibited EAE, and to a lesser extent the human cyclic [Ala^{96}]MBP_{87-99} antagonist peptide.[15] In our in vivo rat studies, EAE was induced by the agonist guinea pig epitope MBP_{72-85} and not by the human MBP_{83-99} agonist peptide. Injections with human agonist peptides caused anaphylactic shock and weak signs of EAE. In those cases, EAE induced by the linear agonist human MBP_{87-99} (**P1**) or counterpart cyclic agonist analogue MBP_{87-99} (**P3**) was not possible to reach maximum clinical score (range 1 to 4), as it is the case with the guinea pig encephalitogenic peptide MBP_{72-85}, which thus was used for the induction of EAE. Rats were able to reach only a low clinical score of 1 when injected with human agonist peptides **P1** and **P3**. However, Lewis rats did not show signs of anaphylactic shock when human EAE antagonist peptides **P2**, **P4**, **P5** were coinjected with encephalitogenic guinea pig epitope MBP_{72-85}. Structure−activity studies demonstrated that residues 91, 96 are critical for inhibitory effects. Thus, replacement in agonist peptide P1 of residues Lys and Pro at positions 91 and 96, which are TCR contact sites and therefore important for encephalitogenicicty, with Arg and Ala, resulted in linear analogue **P2** suppressing guinea pig MBP_{72-85}-induced EAE in Lewis rats. Head-to-tail cyclization of linear antagonist **P2** led to cyclic **P5** which retained antagonist effect in EAE, suggesting a cyclic conformation for linear antagonist in the trimolecular complex. Cyclization between Lys side chain at position 91 with C-terminal

carboxylate led to cyclic **P4** which retained antagonist effect in EAE. Blockade of MBP_{72-85}-induced EAE by the unrelated human linear peptide [Arg^{91}, Ala^{96}]-MBP_{87-99} **P2** and its cyclic analogues **P4**, **P5** could indicate that the mechanism of inhibition is not due to binding competition but rather due to a negative signal by the antagonist which overcomes the agonist response possibly through the activation of antigen specific regulatory T-cells.

EAE antagonist activity alone is not sufficient to provide a pharmacological profile to candidate drugs for treating MS. Other studies were therefore carried out to further evaluate peptide effects in cytokine secretion, CD4+T-cell line proliferation and HLA-DR4 binding which might be useful in the regulation of disease. The present study investigated effects of epitope MBP_{87-99} analogues, (linear **P1**, **P2** and cyclic **P3**, **P4**, **P5** peptides), for their biological activity in vitro, using human PBMC and a human T-cell line from an MS patient. In line with their EAE antagonist activity, linear **P2** and cyclic **P4** and **P5** peptides suppressed proliferation of the T-cell line generated from an MS patient. On the contrary, EAE agonist peptides, linear **P1** and cyclic **P3**, induced proliferation of the T-cell line, showing a similar response to that of the rat and human T-cell populations related respectively to EAE and MS. In addition, their binding ability to HLA-DR4 molecules was determined using HLA-DR4 (DRB1*0401) allele isolated from homogenates by affinity chromatography with the monoclonal antibody L243 generated from MS patients. While guinea pig MBP_{72-85} analogues as expected could not bind to HLA-DR4 molecules, human MBP_{87-99} cyclo analogues **P3**, **P4**, **P5** in this study were found to bind like that of linear counterparts **P1**, **P2**, but with a different degree of capacity. Thus, linear peptides **P1**, **P2** were bound strongly to HLA-DR4, while cyclic counterparts **P3**, **P5** were bound to a lesser degree with comparable affinities. Since alterations were carried out only at the TCR contact sites 89(Phe), 91(Lys), 96(Pro) and not at the HLA contact sites 88(His), 90(Phe), 93-(Ile), variations in affinity may be attributed to conformational changes.[14] Cyclic peptide **P4**, in which the amino group of Lys at 91 participates in an amide bond with the C-terminal carboxyl group, has reduced binding affinity. This is in agreement with altered conformation not allowing optimal binding with HLA groove.

Appropriate cytokine secretion is another important approach in regulating disease. In MS patients, the majority of MBP-specific T-cells isolated during active disease secrete Th1 type cytokines, while during remission, the cytokine profile shifts to IL-4- and IL-10-producing cells.[42] The desirable induction of cytokines should include IL-10 in patients because, as it has been recorded,[43] constitutive presence of IL-4 may lead to anaphylactic shock. The significant role of IL-10 in the induction and function of natural and antigen-induced regulatory T-cells in autoimmune diseases has been again reconfirmed in recent studies.[44] Other studies from crystal structures of superagonist, agonist, and antagonist peptides have demonstrated a loss of H-bond contacts of peptide side chains with the CDR3 loops of the TCR, hence a loss of T-cell activation.[45] Mutation of a large side chain (Asp, Lys) of the peptide that interacts with the TCR to small side chain amino acids (such as Ala, Gly, Ser) can cause antagonism and thus inhibits disease. The analogues to be chosen for their potential use in humans should also induce IL-10 in T-cells from MS patients, suppress T-cell proliferation of T-cells from MS patients, be cyclic for greater stability, and bind to human HLA class II molecules. Therefore, cytokine secretion investigations have been included in the evaluation study of our human MBP_{87-99} linear and cyclic analogues. These studies have shown a specific effect of the cyclic **P4** but not of linear **P1**, **P2** analogues on MS patients' T-cell proliferation, while T-cells from healthy controls did not respond at all to **P4** peptide. In contrast, T-cells from healthy controls responded to all other peptides tested so far, i.e., the nonhuman MBP peptide analogues or modified human MBP peptide analogues or (irrelevant) peptide controls. In particular, TH2 cytokine production by PBMC of healthy controls was increased with linear **P1**, **P2** and cyclic peptides **P3**, **P5** with the exception of **P4** that had no effect on cytokine production by control PBMC. In the case of PBMC cultures from MS patients, the addition of EAE antagonist peptides **P2** and **P4** significantly increased the TH2/TH1 cytokine ratio in 5 of 12 patients with the best effect achieved by **P4**, while it moderately decreased the TH2/TH1 cytokine ratio in 7 of 12 patients. On the contrary, addition of linear **P1** or cyclic **P3**, **P5** peptides to PBMC from MS patients resulted in a decrease of the TH2/TH1 ratio in all patients of the study with the worst effect achieved by EAE agonist peptides **P1** and **P3**. EAE antagonist peptides **P2**, **P4** had a beneficial immunosuppressive effect with high IL-10 and low IFN-γ in 42% of human MS PBMC studied. In addition, the binding of the cyclic antagonist peptides to HLA-DR4 and the effective stability in the presence of lysozymal enzymes makes the antagonist cyclic peptides promising for the use in therapeutic protocols in human trials. Between the two peptides **P2** and **P4**, the side chain Lys^{91}-C-terminal Pro^{99} cyclic analogue **P4** is the most attractive candidate as drug lead, as it scores the best TH2/TH1 cytokine ratio with MS patients PBMC, it has no effect on TH1 or TH2 cytokine production by control PBMC, it suppress CD4 T-cell line proliferation from MS patient, and it is more stable for in vivo use.

Conclusion

A rationally designed amide-linked head-to-tail cyclic analogue, cyclo(87−99)MBP_{87-99}, was synthesized and found to induce EAE in Lewis rats and proliferation of CD4 T-cell line from MS patients. On the contrary, side chain 91 to C-terminal tail and head-to-tail cyclic analogues **P4** and **P5**, with alterations at positions 91 and 96 which are TCR contact sides, were found to suppress EAE and CD4 T-cell line proliferation. Among cyclic analogues **P3**, **P4**, **P5**, analogues which all are bound to HLA DR4 and all are stable to lysosomal degradation compared to linear counterparts, analogue **P4** is the most promising for development since (i) it has no effect on TH1 or TH2 cytokine production by control PBMC, (ii) it scores the best TH2/TH1 cytokine ratio with MS patients PBMC, and (iii) being cyclic, it is stable for in vivo use.

Experimental Procedures

(1) Synthesis of Cyclic Peptide P3. *(i) Solid-Phase Peptide Synthesis of Linear Precyclic Analogue: Val-His(Trt)-Phe-Phe-Lys(Boc)-Asn-Ile-Val-Thr(tBu)-Pro-Arg(Pbf)-Thr(tBu)-Pro.* The linear protected peptide was prepared on the acid-sensitive 2-chlorotrityl chloride resin (CLTR-Cl) using Fmoc/tBu methodology. The first N^{α} -Fmoc (9-fluorenylmethyloxycarboxyl)-protected amino acid [Fmoc-Pro-OH (4.5 mmol, 1.25 g)] was esterified on the resin (3 g, 1.5 mmol Cl^{-}/g resin) in the presence of diisopropylethylamine (DIPEA) (13.5 mmol, 2.25 mL) in DCM using a method described previously.[22] The substitution was found to be 0.65 mmol amino acid/g resin. First, the protected linear peptide Val-His(Trt)-Phe-Phe-Lys-(Boc)-Asn-Ile-Val-Thr(tBu)-Pro-Arg(Pbf)-Thr(tBu)-Pro-CLTR (4.15 g) was synthesized using Fmoc-Thr(tBu)-OH, Fmoc-Arg-(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Ile-OH, Fmoc-Asn-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH. After deprotection of Fmoc, coupling was carried out in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in DMF. In each case the Fmoc protecting group was removed by treatment with piperidine (20% in DMF, 2 × 15 min). The Kaiser test and thin-layer chromatography (TLC) in *n*-butanol/acetic acid/water (4:1:1) (BAW) as elutant verified the completeness of each coupling or Fmoc deprotection. The protected peptide-resin was treated with the splitting mixture DCM/HFIP (7/3, 15 mL) for 3 h at room temperature to remove peptide from the resin. The mixture was filtered off and the resin was washed with the splitting mixture (×2) and DCM (×3). The solvent was removed on a rotary evaporator and the obtained oily product was precipitated by cold and dry diethyl ether as a white solid.

(ii) Cyclization Procedure of Protected Linear Peptide: Val-His(Trt)-Phe-Phe-Lys(Boc)-Asn-Ile-Val-Thr(tBu)-Pro-Arg(Pbf)-Thr(tBu)-Pro. To a solution of the above linear protected peptide (200 mg, 0.09 mmol) in dry DMF (15 mL) were added 2,4,6-collidine (0.072 mL, 0.54 mmol) and 1-hydroxy-7-azabenzotriazole (36.8 mg, 0.27 mmol). The solution was then added dropwise to a stirred solution of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) (86.7 mg, 0.27 mmol) in dry dimethylformamide (80 mL) for 2 h. Solution was stirred for 4 h. The reaction was followed by the ninhydrin test on TLC (BAW). The solvent was removed from the reaction mixture under reduced pressure affording a light yellow oily residue. The cyclic protected peptide was precipitated from H_2O and was dried in vacuo for 12h (Scheme 1).

(iii) Preparation of Final Cyclic Analogue P3: Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro. The protected cyclic peptide was treated with the deprotection mixture DCM/TFA/ethanedithiol/anisole (32/65/2/1) for 4 h at room temperature. The resulting solution was concentrated under vacuum to a small volume (0.5 mL). The final free cyclic peptide was precipitated as a light-yellow amorphous solid by the addition of diethyl ether, filtered, and then was dried in vacuo for 12 h (purity >80%). The final crude products were further purified using RP-HPLC.

(iv) Analysis, Detection, and Identification: HPLC/TLC/ESIMS. Preparative HPLC for cyclic analogue was performed with a Waters system equipped with a 600 controller system using a Lichrosorb RP-18 reversed-phase semipreparative column (250 × 10 mm) with 7 μm packing material. Separations were achieved with a stepped linear gradient of acetonitrile (AcN) (0.08%TFA) in water (0.08% TFA) over 50 min at a flow rate of 3 mL/min. Peptide purity was assessed by analytical RP-HPLC, thin-layer chromatography (TLC) (BAW, 4:1:1), and mass spectrometry (ESI-MS). The RP-HPLC/t_R was found to be 12.04 min and the ESI-MS ($M + H^+$) to be 1538.

(v) Conditions of RP-HPLC: column: Nucleosil C18, 250 × 4.0 mm, gradient separation: 20%B to 100%B in 27 min (B: TFA/AcN 0.08%).

(2) Peptides Subjected to Biological Tests. P1: linear agonist MBP_{87-99} peptide. (VHFFKNIVTPRTP).

Table 3. Dihedral Angles Phi, Psi, Omega of All Residues in the 3-D Model of Linear MBP_{87-99} Epitope and Experimental Coupling Constant (*J*) for Phi

amino acid	phi	psi	omega	*J* coupling constant (phi) Hz
Val1	-	−174	−169.4	
His2	−136.1	170.7	177.7	8.08
Phe3	−34.3	−55.9	−176.3	7.84
Phe4	−70.9	90.8	−176.8	7.80
Lys5	−121.2	19.9	167.1	8.80
Asn6	−80.9	124.1	−29.7	7.24
Ile7	−104.6	−130.1	166.5	9.00
Val8	−63.9	−16.1	168.6	8.64
Thr9	−122.8	135.2	−8.2	6.76
Ala10	−72.4	133.3	167.8	
Pro11	−90.2	19.1	172.5	8.04
Thr12	−133.5	97.2	−22	6.84
Pro13	−79	- - -	- - -	

P2: linear antagonist $[Arg^{91}Ala^{96}]MBP_{87-99}$ peptide (VHFFRNIVTARTP).

P3: cyclic agonist cyclo(87−99)MBP_{87-99} peptide (VHFFKNIVTPRTP; cyclized between Val^{87}-Pro^{99}).

P4: cyclic antagonist cyclo(91−99)$[Ala^{96}]MBP_{87-99}$ peptide (VHFFKNIVTARTP; cyclized between Lys^{91}-Pro^{99}).

P5: cyclic antagonist cyclo(87−99)$[Arg^{91}Ala^{96}]MBP_{87-99}$ peptide (VHFFRNIVTARTP; cyclized between Val^{87}-Pro^{99}).

(3) NMR Spectroscopy. Linear agonist peptide (**P1**) and its antagonist (**P2**) were dissolved in DMSO-d_6 giving a concentration of 3 mM. Variable temperature experiments were performed on a Bruker AMX-400 instrument, from 295 to 320 K, to calculate the temperature coefficients of chemical shifts of the NH protons, which are helpful in certain cases. Homonuclear two-dimensional $^1H-^1H$ TOCSY (100 ms mixing time) and ROESY (200 ms evolution time) experiments were performed at 300 K, using standard pulse sequences.

(4) In Vivo Induction/Suppression of EAE. Female Lewis rats were injected subcutaneously in the hind footpads as described [15] with peptide analogues **P1**−**P5** and emulsified in complete Freund's adjuvant (CFA). Clinical EAE was graded on a scale of 0−4. Rats developed anaphylactic shock and the clinical score was 1, when injected with the agonist peptides **P1** and **P3**.

(5) Effect of Peptides on Cytokine Secretion by PBMC Derived from MS Patients. Twelve patients with definite remitting-relapsing MS and five healthy adults were studied. Four MS patients were male and eight female with median age 36.9 (range 17 to 66 years) and median disease duration 6.25 years (range 0 to 18 years). The controls were two male and three female with median age 30 years (range 24 to 45 years). MS patients presented to the Neurology Clinic of Patras University Hospital (PUH). Samples of heparinized blood (5−10 mL) were drawn from patients and controls. Informed consent was obtained from each participating patient and control. PUH abides by the Helsinki declaration on ethical principles for medical research involving human subjects. PBMC were prepared by centrifugation over a Ficoll-Paque gradient (Pharmacia, Sweden). Cells (10^6/group) were cultured for 72 h in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD), containing 10% fetal bovine serum and 1% penicillin/streptomycin (CM), in the presence or absence of peptides **P1**−**P5**, at 7 nM concentration (optimal peptide concentration as assessed by dose−response experiments, data not shown). The cells were counted using a Sysmex NE-8000 counter (Kyoto, Japan), and their viability was estimated by trypan blue exclusion. At the end of the culture period, supernatants were collected and their cytokine contents were measured by ELISA.[46] The following cytokines were assessed: human (h) IL-2 by ELISA from Roche Diagnostics GmbH (Mannheim, Germany), hIFN-γ by ELISA from R&D Systems Quantikine™ (Minneapolis, USA), hIL-4 and hIL-10 by ELISA from Endogen Inc. (Woburn, MA).

(6) Generation of MBP_{80-99} Specific CD4+T-Cell Line. Peptide specific T-cell lines were generated using PBMC from one MS patient, by a modified split-well technique described

previously.[47] Briefly, PBMC were seeded at a concentration of 2×10^5 cells/well in CM into 96-well plates (Nunc, Roskilde, Denmark) and stimulated with 5 μg/mL of MBP_{80-99} peptide. Seven days later, 10U/mL human-recombinant IL-2 (rIL-2) (R&D Systems, Minneapolis, MN) was added to each well. Seven days later, cells were washed and resuspended in 200 μL of CM. 50 μL of the cell suspension was transferred into two adjacent wells of a separate 96-well microtiter plate along with 150 μL of CM containing 1×10^5 autologous irradiated (3000 rad) PBMC. One well was stimulated 5 μg/mL of MBP_{80-99} peptide. Seventy-two hours later 0.5 μCi of ^{3}H-thymidine (Amersham, Milan, Italy) was added to each well, and after an additional 6 h, ^{3}H-thymidine incorporation was measured in a scintillation counter (Microbeta Plus, Walllac, Milan, Italy). Wells showing a stimulation index (SI = cpm of antigen-stimulated culture/cpm of unstimulated culture) > 2 were considered positive. The frequency of MBP_{80-99}-specific T-cells was estimated by calculating the number of positive wells for each seeding. Peptide-reactive cultures were further expanded by cyclic stimulation with autologous irradiated PBMC in the presence of MBP_{80-99} peptide and rIL-2.

In vitro proliferative responses were also determined for linear and cyclic MBP_{87-99} peptide analogues (**P1**−**P5**), using the MBP_{80-99}-specific T-cell line. 1×10^4 T-cells were mixed with 5×10^4 autologous irradiated PBMC in CM or 0.001−10μg/mL **P1**−**P5** peptides. After 72 h, ^{3}H-thymidine incorporation was measured as described above.

(7) Binding of P1−P5 to HLA-DR4 (DRB1*0401)-HPSEC Binding Assay. EBV transformed homozygous human B cell lines BSM (DRB1*0401), and HTC-Lan (DRB1*1501, DRB5*0101) were used for isolating MHC. BSM and HTC-Lan cell pellets were lyzed by nonidet P-40 and HLA-DR isolated from homogenates by affinity chromatography using the monoclonal antibody L243.[48] The purity of the preparation was checked by SDS−PAGE, HPSEC, and western blotting (not shown). HLA binding assays for linear and cyclic MBP_{87-99} analogues were carried out as follows:

Solubilized HLA-DR4 (0.13 uM) was incubated for 48 h at 37 °C with the N-terminally 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-labeled influenza matrix protein (306−318) peptide (AMCA-HA306−318-peptide) dissolved in 150 mM sodium phosphate, pH 5.5, containing 15% acetonitrile, 0.1% Zwittergent-12 (Calbiochem), and a cocktail of protease inhibors (0.2 mM PMSF, 5 μM leupeptin, 10 μM pepstatin, and 1 μM chymostatin). Competition assays were performed in a 1.5 μM solution of AMCA peptide. As competitors, different MBP peptides (**P1**−**P5**) were added in 5-fold, 20-fold, and 50-fold excess compared to the AMCA-peptide.[29] All samples were analyzed on a Pharmacia Superdex 75 HR 5/20 high performance gel filtration column, essentially as described.[29] The column was operated at a flow rate of 0.4 mL/min using the HPSEC buffer, pH 6.0. The effluent passed through a Shimadzu fluorescence spectrometer (350/450 nm) and a Merck ultraviolet detector (214 nm) set in series. Fluorescence and UV signals eluting with the HLA-DR dimers were recorded by a model D 2500 integrator (Merck-Hitachi). The effluent passed through a fluorescence spectrometer (350/450 nm) and an ultraviolet detector (214 nm) set in series.

(8) Stability of Cyclic/Linear Peptides. Peptides **P1**−**P5** were digested in a time course with different lysosomal enzymes. Lysosomal fractions were isolated from BSM cells by differential centrifugation and hypotonic lysis.[48] Cathepsins B, D, and H were obtained from Sigma. Peptides (2.5 μg) were dissolved in 50 μL of the different substrate buffers and incubated with enzymes at 37 °C.[49,50] Between 0 and 90 min, 15 μL of solution was taken and quenched with 5 μL of 20% TFA at 4 °C. Samples were analyzed by analytical HPLC using a Vydac C_{18} 5 μm column with a linear gradient of buffer A (0.05%TFA/H_2O) and buffer B (80% acetonitrile/0.05%TFA/H_2O) from 5 to 80% B in 40 min at 214 nm. The relative concentration of each compound was estimated from the area under the sample peak.

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