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)MBP87–99], of the human immunodominant MBP87–99 epitope, was designed based on ROESY/NMR distance information and modeling data for linear epitope 87–99, taking into account T-cell (Phe89, Lys91, Pro96) and HLA (His88, Phe90, Ile93) 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 MBP87–99 epitope peptide. The mutant cyclic peptides, the cyclo(91–99)[Ala96]MBP87–99 and the cyclo(87–99)[Arg91Ala96]MBP87–99, 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., MBP1–11, MBP7–99, MBP84–102, MBP97–106, and MBP83–99), 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 (IFN-γ) immune response toward TH2 (IL-10).13 A linear APL of the immunodominant epitope MBP83–99 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 MBP87–99 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...
proteolytic hydrolysis or nonpeptide mimetics of the parent molecule for clinical purposes, it is necessary to use more stable peptides as therapeutic entities, in general, is limited because of their sensitivity to proteolytic enzymes. Therefore, to address the need for a more stable peptide-based MS therapy, as it is still in its infancy. Efforts to design semimimetics of MBP72–85 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 MBP72–85 peptide (QKSQRSQDENPV) induced EAE; cyclization of MBP72–85 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 [Ala81]MBP72–85 which inhibited EAE when coinjected with the linear or cyclic agonist peptide.19,20 In addition, human MBP7–99 (VHFFKNIVTRKPRTP) linear epitope induced EAE in rats, which was inhibited by the antagonist linear peptide [Arg91, Ala96]MBP7–99 (VHFFRNIVTARTP) or antagonist cyclic peptide, cyclo (87–99)[Arg91, Ala96]MBP87–99. Furthermore, coinjection of linear guinea pig MBP72–85 peptide analogues P3–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 spectroscopy 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.

Figure 1. Low energy conformers of MBP87–99 peptide analogues. a, linear agonist MBP87–99 (P1); b, linear antagonist [Arg91, Ala96]MBP87–99 (P2); c, cyclic agonist cyclo(87–99)MBP87–99 (P3); d, cyclic antagonist, cyclo(91–99)[Ala96]MBP87–99 (P4) and e, cyclic antagonist, cyclo(87–99)[Arg91, Ala96]MBP87–99 (P5).
agonist peptide with linear or cyclic human [Arg⁹¹, Ala⁹⁶]MBP₇₉–₉₉ antagonist peptide inhibited EAE in Lewis rats.¹⁵,²¹

Herein, we demonstrate that rationally designed cyclic peptide P₃, based on human MBP₇₉–₉₉, induces EAE in rats, binds to HLA-DR4, which is the first to be reported for cyclic MBP peptide mimics, and increases the common amino acid residues in both epitopes, thus confirming that a backbone turn in the His²-Ile⁷ segment. Also, a weak Ile⁷-Thr¹₂ cross-peak was observed, indicating a bend in the C-terminal segment. Strong HN(i)–HN(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 HN(i)–HN(i+2) or a weak HN(i)–HN(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 HN(i)–HN(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 HN(i)–HN(i+1) region of the antagonist. This finding was confirmed by a weak Val¹ HN cross-peak. In a second turn is expected in the His²-Ile⁷ segment. Strong HN(i)–HN(i+1) cross-peaks were observed, besides the other HN(i)–HN(i+1) and HN(i)–HN(i+2) 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).

### 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 Hα–Hα region of the two-dimensional TOCSY experiments of the two peptides. Using row or column projections for all the cross-peaks in the Hα–Hα 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.²²a,b

In particular, examining the ROESY spectrum of the agonist, the following medium HN(i)–HN(i+1) cross-peaks were observed: His²–Phe³, Phe³–Phe⁴, Phe⁴–Lys⁶, Lys⁶–Asn⁶, Asn⁶–Ile⁷, Val⁸–Thr⁹, and Arg¹ⁱ–Thr¹₂, indicating a backbone turn in the His²-Ile⁷ segment. Also, a weak Ile⁷-Thr¹₂ cross-peak was seen, indicating a bend in the C-terminal segment. Strong HN(i)–HN(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 HN(i)–HN(i+2) or a weak HN(i)–HN(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 HN(i)–HN(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 Val¹–His²–Phe³–Phe⁴–Lys⁶–Asn⁶–Ile⁷, which is also confirmed by a weak Val¹ Hα–Lys⁶ Hα cross-peak. A second turn is expected in the segment Val¹–Thr³–Pro¹⁰–Arg¹¹–Thr¹₂–Pro¹³, due to the existence of the weak Ile⁷ Hα–Thr¹₂ Hα cross-peak. Examining the ROESY spectrum of the antagonist, the medium Ala¹⁰ Hα–Arg¹¹ Hα and the strong Thr⁹ Hα–Ala¹⁰ Hα cross-peaks were observed, besides the other HN(i)–HN(i+1) and HN(i)–HN(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)MBP₇₉–₉₉ (P₃). For the synthesis of the cyclic peptide P₃, acid-sensitive 2-chlorotrityl chloride resin (CLTR-CI), appropriate side chain protection, and

### Table 1. ¹H NMR Assignments, Chemical Shifts (δ, ppm) and NH Temperature Coefficients (∆∆ΔT) of Agonist MBP₇₉–₉₉ and Antagonist [Arg⁹¹, Ala⁹⁶]MBP₇₉–₉₉ in DMSO-d₆ at 300 K

<table>
<thead>
<tr>
<th>amino acid</th>
<th>NH</th>
<th>α</th>
<th>β</th>
<th>other</th>
<th>∆∆ΔT (ppb/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val 1</td>
<td>-</td>
<td>3.59</td>
<td>1.96</td>
<td></td>
<td>y: 0.83</td>
</tr>
<tr>
<td>His 2</td>
<td>8.60</td>
<td>4.64</td>
<td>2.96, 2.89</td>
<td>2H: 8.92, 4H: 7.22</td>
<td>3.88</td>
</tr>
<tr>
<td>Phe 3</td>
<td>8.20</td>
<td>4.55</td>
<td>3.00, 2.75</td>
<td>ring: 7.29–7.11</td>
<td>4.11</td>
</tr>
<tr>
<td>Phe 4</td>
<td>8.34</td>
<td>4.58</td>
<td>3.05, 2.84</td>
<td>ring: 7.29–7.11</td>
<td>5.01</td>
</tr>
<tr>
<td>Lys 5</td>
<td>8.13</td>
<td>4.33</td>
<td>1.64</td>
<td></td>
<td>y: 1.31, δ: 1.53, ε: 2.75, γ: 7.70</td>
</tr>
<tr>
<td>Asn 6</td>
<td>8.27</td>
<td>4.61</td>
<td>2.53, 2.41</td>
<td>NH: 7.39, 6.95</td>
<td>5.39</td>
</tr>
<tr>
<td>Ile 7</td>
<td>7.74</td>
<td>4.23</td>
<td>1.74</td>
<td>y: 1.38, γ: 1.07, δ: 0.81</td>
<td>3.42</td>
</tr>
<tr>
<td>Val 8</td>
<td>7.81</td>
<td>4.23</td>
<td>1.96</td>
<td>y: 0.83, 0.79</td>
<td>3.66</td>
</tr>
<tr>
<td>Thr 9</td>
<td>7.93</td>
<td>4.39</td>
<td>3.85</td>
<td>y: 1.12</td>
<td>7.33</td>
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<td>2.13, 1.91</td>
<td>y: 1.87, δ: 3.77, 3.61</td>
<td>-</td>
</tr>
<tr>
<td>Arg 11</td>
<td>8.00</td>
<td>4.30</td>
<td>1.68</td>
<td>y: 1.45, δ: 3.08, ε: 7.46</td>
<td>4.17</td>
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<td>Thr 12</td>
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<td>4.32</td>
<td>3.92</td>
<td>y: 1.14</td>
<td>6.40</td>
</tr>
<tr>
<td>Pro 13</td>
<td>-</td>
<td>4.20</td>
<td>2.15, 1.91</td>
<td>y: 1.84, δ: 3.78, 3.64</td>
<td>-</td>
</tr>
</tbody>
</table>
Cyclo(87–99) side chain protection (except for the termini NH2 and tBu for Ser, Thr, Asp, Glu, and Boc for Lys. The use of amino acids were protected with Trt for His, Pbf for Arg, without side products. In particular the side chains of these reagents induced fast, quantitative reactions achieved using TBTU/HOAt and collidine as base.15,19,20

Synthetic Procedure for Cyclic Analogue: Scheme 1.

Induction of EAE with Cyclic MBP97–99 (P3). We have previously demonstrated that the linear human MBP97–99 peptide induces weak encephalitogenic activity in Lewis rats.15 Similarly, the cyclic MBP97–99 analogue P3 induced weak EAE (clinical score 1). The use of human MBP97–99 agonist peptides in Lewis rats results in anaphylactic shock and weak signs of EAE. On the contrary, guinea pig (gp) epitope MBP72–85 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 gpMBP72–85. Clinical signs of EAE induced by MBP97–99 or by MBP72–85 were completely suppressed by head-to-tail cyclic analogue P5 and to a lesser degree by the Lys91 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 coinfected with MBP72–85 or MBP97–99 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 MBP90–99, P1) was tested for proliferative activity to P1–P5 peptides. The linear MBP97–99 peptide (P1) and cyclo(87–99)MBP97–99 (P3) induced T-cell proliferation. In contrast, the antagonist cyclic peptides, cyclo(91–96)[Ala96]MBP97–99 (P4) and cyclo(87–99)[Arg91, Ala96]MBP97–99 (P5), inhibited proliferation of the T-cell line as did the linear antagonist [Arg91, Ala96]MBP97–99 (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 ×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.
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 HA306–318-peptide and immunoaffinity-purified HLA-DR isolates from EBV-transformed cells. As competitors, the MBP peptides were used in different concentrations. Guinea pig epitope MBP72–85 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.

Stability of Cyclic Peptides with Lysosomal Enzymes. To determine the stability of cyclic versus linear MBP₈₇–₉₉ 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 5b). Cathepsin D is an aspartic protease which cleaves predominantly between hydrophobic amino acids, and in MBP between Phe₈₉–Phe₉₀.³¹ Furthermore, digestion with the exopeptidases — Cathepsin B (peptidyldepeptidase, 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 5c). It is clear,
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 Val1 and the α-NH proton of Arg1, which is observed in agonist’s molecule, together with the long-range ROE connectivity between the α-CH proton of Val1 and the β-CH proton of Thr12, 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 η-NH proton of Arg97 and the γ-CH$_3$ proton of Thr95 connectivity with the ring protons of Phe89 or Phe90, not possible to be differentiated as overlapping (Figure 6). Molecular modeling based on critical inter-residue 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 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. 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...
antagonist activities for cyclic analogues cyclo(91–99)-[Ala96]MBP87–99 (P4) and cyclo(87–99)[Arg91, Ala96]MBP87–99 (P5) designed based on the human MBP antagonist analogue cyclo(87–99) [Arg91, Ala96] (MBP87–99) 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 MBP72–85. The immunodominant epitope identified in humans is MBP85–99 or MBP87–99. 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 mimic 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 MBP72–85 and 91, 96 in human MBP83–99, 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,23,24 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 MBP72–85, 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 MBP72–85 peptides induced EAE.19 Coinjection of linear guinea pig MBP72–85 agonist peptide with human cyclic [Arg91, Ala96]MBP87–99 antagonist peptide inhibited EAE.15 In addition, coinjection of linear guinea pig MBP72–85 agonist peptide with the cyclic human [Arg91, Ala96] MBP87–99 antagonist peptide inhibited EAE, and to a lesser extent the human cyclic [Ala96]MBP87–99 antagonist peptide.15 In our in vivo rat studies, EAE was induced by the agonist guinea pig epitope MBP72–85, and not by the human MBP83–99 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 MBP87–99 (P1) or counterpart cyclic agonist analogue MBP87–99 (P3) was not possible to reach maximum clinical score (range 1 to 4), as it is the case with the guinea pig encephalitogenic peptide MBP72–85, 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 MBP72–85. 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 encephalitogenicity, with Arg and Ala, resulted in linear analogue P2 suppressing guinea pig MBP72–85-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 MBP72–85-induced EAE by the unrelated human linear peptide [Arg91, Ala96], MBP87–99 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 MBP87–99 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 (DBR1*0401) allele isolated from homogenates by affinity chromatography with the monoclonal antibody L243 generated from MS patients. While guinea pig MBP72–85 analogues as expected could not bind to HLA-DR4 molecules, human MBP87–99 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 MBP87–99 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 Lys91-C-terminal Pro99 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)MBP87–99, 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(Trr)-Phe-Phe-Lys(Boc)-Asn-Ile-Val-Thr(Bu)-Pro-Arg(Pbf)-Thr(Bu)-Pro. The linear protected peptide was prepared on the acid-sensitive Rink-CH2Cl resin (ClTRT-CI) using Fmoc/Bu methodology. The first N-terminus Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acid (Fmoc-Pro-OH (4.5 mmol, 1.25 g)) was esterified on the resin (3 g, 1.5 mmol Cl/f/g resin) in the presence of diisopropylethylamine (DIEA) (13.5 mmol, 2.25 mL) in DCM using a method described previously.21 The substitution was found to be 0.65 mmol amino acid/g resin. First, the protected linear peptide Val-His(Trr)-Phe-Phe-Lys(Boc)-Asn-Ile-Val-Thr(Bu)-Pro-Arg(Pbf)-Thr(Bu)-Pro-CLTRT (4.15 g) was synthesized using Fmoc-Thr(Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Thr(Bu)-OH, Fmoc-Val-OH, Fmoc-Ile-OH, Fmoc-Asn-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-His(Trr)-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 DCM using a method described previously.22 The protected 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 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 J = H–H TOCSY (100 ms mixing time) and ROESY (200 ms evolution time) experiments were performed at 300 K, using standard pulse sequences.

(2) Peptides Subjected to Biological Tests. P1: linear agonist MBP87–99 peptide. VHHFKNVTARP.

| Table 3. Dihedral Angles Phi, Psi, Omega of All Residues in the 3-D Model of Linear MBP87–99 Epitope and Experimental Coupling Constant (J) for Phi |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| amino           | phi             | psi             | omega           | J coupling      |
| acid            | constant (Hz)   | constant (Hz)   | constant (Hz)   | constant (Hz)   |
| VAl1            | -174           | 169.4           | 8.08            |
| His2            | 170.7          | 177.7           | 7.84            |
| Phe3            | 55.9           | 176.3           | 7.80            |
| Phe4            | 90.8           | 176.8           | 7.80            |
| Lys5            | 19.9           | 167.1           | 8.08            |
| Asn6            | 124.1          | 29.7            | 7.24            |
| His7            | 120.1          | 166.6           | 9.05            |
| Val8            | 168.6          | 8.64            |
| Thr9            | 153.2          | 8.2             |
| Thr12           | 133.3          | 8.4             |
| Pro11           | 18.1           | 7.22            |
| Pro13           | -79            | -                |


P3: cyclic agonist cyclo(87–99)MBP87–99 peptide (VHHFKNVTARP; cyclic between Val87-Pro99).


P5: cyclic antagonist cyclo(87–99)[Arg6Ala36]MBP87–99 peptide (VHHFKNVTARP; cyclic between Val87-Pro99).

(3) NMR Spectroscopy. Linear agonist peptide (P1) and its antagonist (P2) were dissolved in DMSO-d6 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 J = H–H 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 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. For MS patients were male and eight female with median age 36.9 years (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 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 36.9 years (range 24 to 45 years). MS patients presented to the Neurology Clinic of Patras University Hospital (PUH). Samples of heparinized blood (5 mL) were collected and their cytokine contents were measured by ELISA from R&D Systems Quantikine.

(6) Generation of MBP80–98 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. Briefly, PBMC were seeded at a concentration of 2 \times 10^6 cells/well in CM into 96-well plates (Nunc, Roskilde, Denmark) and stimulated with 5 \mu g/mL of MBP99-99 peptide. Seven days later, 100\mu L 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 \mu L of CM. 50 \mu L of the cell suspension was transferred into two separate wells of a 96-well microtiter plate along with 150 \mu L of CM containing 1 \times 10^4 autologous irradiated (3000 rad) PBMC. One well was stimulated with 5 \mu g/mL of MBP99-99 peptide. Seventy-two hours later 0.5 \mu Ci of \textsuperscript{3}H-thymidine (Amersham, Milan, Italy) was added to each well, and after an additional 6 h, \textsuperscript{3}H-thymidine incorporation was measured in a scintillation counter (Microbeta Plus, Wallac, Finland).

Wells showing a stimulation index (SI = cpm of antigen-stimulated culture/cpm of unstimulated culture) > 2 were considered positive. The frequency of MBP99-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 MBP99-99 peptide and rIL-2.

In vitro proliferative responses were also determined for linear and cyclic MBP99-99 peptide analogues (P1-P5), using the MBP99-specific T-cell line. 1 \times 10^5 T-cells were mixed with 5 \times 10^4 autologous irradiated PBMC in CM or 0.001–10 ng/mL P1-P5 peptides. After 72 h, \textsuperscript{3}H-thymidine incorporation was measured as described above.

(7) Binding of P1-P5 to HLA-DR4 (DRB1*0401). HPSEC Binding Assay. EBV transformed homogenous human B cell lines BSM (DRB1*0401), and HTC-Lan (DRB1*1501, BSM) were used for isolation of HLA-DR4 and HLA-DR1; BSM and Lan cell pellets were lyzed by nonidet P-40 and HLA-DR4 isolated from homogenates by affinity chromatography using the monoclonal antibody L243. The purity of the preparation was checked by SDS-PAGE, HPSEC, and western blotting (not shown). HLA binding assays for linear and cyclic MBP99-99 analogues were carried out as follows:

Sized HLA-DR4 (0.13 mM) was incubated for 48 h at 37 \degree C with the N-terminals 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% trifluoroacetic acid (TFA)/H2O and buffer B (80% acetonitrile/0.05%TFA/H2O) 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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References

The image contains a section of a scientific article from the Journal of Medicinal Chemistry, discussing the synthesis and biological effects of cyclic MHC II analogues of myelin basic protein (MBP) epitope 72-85, and their implications in the design of non-peptide antagonists for autoimmune diseases such as experimental allergic encephalomyelitis (EAE) and multiple sclerosis.

Key points:
1. The design and synthesis of cyclic analogues of the MBP epitope 72-85, which is known to induce experimental allergic encephalomyelitis (EAE) in guinea pig models.
2. The importance of the C-terminal aromatic residue and the primary amino group for activity.
3. The role of the Leu-Arg motif and the alpha-amino acid residues in determining the activity of the cyclic analogues.
4. The potential of these cyclic analogues as a rationally designed therapeutic approach for the treatment of autoimmune diseases.

This article highlights the ongoing efforts to develop novel therapeutic strategies for autoimmune diseases by targeting MHC II molecules and their peptide ligands.