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A Novel Aza-MBP Altered Peptide Ligand for the Treatment of Experimental Autoimmune Encephalomyelitis

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Abstract

Myelin basic protein (MBP) is a major target of T cells in lesions of multiple sclerosis (MS) patients and its animal model, experimental autoimmune encephalomyelitis (EAE). Interactions between the major histocompatibility complex II containing antigenic peptides and the T cell receptor activate CD4⁺ T cells that perpetuate EAE and MS. Previously reported data has shown that treating with an altered peptide ligand (APL) in which the normal antigenic peptide sequence of MBP has been slightly changed at T cell contact positions is helpful in reducing disease in both rodents and humans. The use of natural peptides, which are susceptible to protease degradation, requires high concentrations that can create hypersensitivity reactions. Our hypothesis is that APL containing aza substitutions, CH(R)-N → N(R)N, could lead to improved protease resistance, reduced clinical disease scores, and a shift in T cell profile. In this study, several aza-APLs and control peptides were synthesized and screened for the best aza-APL candidate (3aza-APL) based on dissociation half-time from major histocompatibility complex (MHC) class II, induction of IL-2 response, and resistance to degradation by proteases. The efficacy was then tested *in vivo*. Results indicate that 3aza-APL is superior to currently available APLs in terms of protease resistance and disease suppression in EAE mice. The 3aza-APL induced anti-inflammatory immune responses by altering key transcription factors and cytokine genes which regulate T cell subpopulations. These data suggest that the novel 3aza-APL has increased protease resistance property and is effective in reducing clinical and physiological signs of disease in EAE animals.

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Keywords

Multiple sclerosis; Experimental autoimmune encephalomyelitis; Altered peptide ligand; MHC II; T cell receptor

Introduction

The immunological synapse is formed by the T cell receptor (TCR) and major histocompatibility complex class II (MHC II) with a peptide displayed in the binding groove along with accessory binding molecules and this complex is necessary to activate a specific T cell [1]. The peptide that lies inside this complex, the component of a protein processed inside the cell and bound to a specific MHC II molecule, will bind to a specific TCR. This peptide is the antigenic sequence of a target protein. Earlier studies have shown that altering the sequence of this peptide can influence the binding kinetics of the TCR, either increasing or decreasing the affinity, and thereby alter the response from the target T cell [2]. In fact, the TCR can recognize ligand peptides that have no sequence homology, thus opening the possibility for development of APLs with no potential for cross reaction [3]. This altered response can result in immune modulation and change the default inflammatory response [4]. Certain changes have resulted in an alteration from an inflammatory response to T helper 2 (Th2) and/or T regulatory (Treg) response [5].

The concentration of antigen influences the avidity of the TCR present in certain populations of T cells. However, T cells with less antigen may also result in a population with strong avidity for an antigen while those with more antigen result in a population with a low avidity TCR [6]. This observation demonstrates the importance of bioavailability in APL design and the population of reactive cells that may result. Antigen specific therapy has been attempted with a number of myelin protein sequences [7]. In fact, this strategy has been employed to alter the disease course of EAE and has also been attempted in MS patients with altered MBP peptide [8, 9]. These clinical trials were promising but ultimately proved disappointing when unintended hypersensitivity reactions occurred. The hypersensitivity responses were theorized to be due to development of an allergy to the APL caused by a shift to a Th2 bias after loss of self- tolerance. Additionally, when the trial was performed again, this time with doses spaced farther apart in order to decrease hypersensitivity reactions, no clinical benefit was seen above standard therapy [10]. The low bioavailability of the test APL because of protease activity and large frequent doses required for effective treatment possibly contributed to sensitizing the patients to the allergic reactions [9]. However, the antigen directed approach is still viable and APLs are the most specific version of this strategy, despite the hypersensitivity side effects. From these studies it is clear that further optimization and improvements to the APL to increase bioavailability is needed to result in a more favorable therapeutic outcome.

Peptides in the body, either native signaling peptides or peptides used as drugs, are subject to degradation by non-specific proteases on the cell surfaces [11]. Therefore, changes to a peptide sequence in order to create an APL with increased protease resistance may result in a peptide with increased bioactivity. The increased protease resistance may allow for a

reduced dose while still maintaining an immunomodulatory effect ultimately resulting in an APL with less potential for hypersensitivity side reactions. This study sought to address some of the short comings of the bioavailability recognized in previous studies on APL [12].

Aza-amino acids, an amino acid with nitrogen substituted for the α -carbon, have been utilized to create APLs with some of the properties that contribute to bioavailability such as protease resistance [13]. Studies have demonstrated aza peptide resistance to amino- and carboxypeptidases, and they have also been used as prodrugs [14]. Here, several aza-APLs that mimic MBP were investigated to see if an increased bioavailability could be created while still maintaining bioactivity in order to reduce EAE disease *in vivo*.

Materials and Methods

Cell Culture and Reagents

The T cell activation studies utilized a mouse L929 line of fibroblast like cells that have been transfected to express I-A^u MHC II protein [15] and a T cell hybridoma line [16] that specifically expresses the 172.10 TCR specific for rat MBP Ac1–11 peptide. The L929 cells were maintained in Dulbecco's modified Eagle F12 media (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin and 1 μ g/ml streptomycin (Gibco) and 10% FCS (Hyclone, Logan, UT) at 37°C in a 5% CO₂ atmosphere (Thermo Fisher Scientific, Waltham, MA) and grown to desired confluency for each of the assays performed. The 172.10 cells were maintained in RPMI 1640 media (Gibco) supplemented with 100 U/ml penicillin and 1 μ g/ml streptomycin (Gibco) and 10% FCS (Hyclone, Logan, UT) at 37°C in a 5% CO₂ atmosphere (Thermo Fisher Scientific, Waltham, MA) and maintained between 1×10^6 and 5×10^6 cells per ml of media.

Peptide Synthesis and Labeling

Peptide synthesis grade reagents were purchased from Advanced Chemtech (Louisville, KY), and peptide components were obtained from Peptides International (Louisville, KY). The native and altered MBP peptides were synthesized using rink amide resin (Novabiochem, Darmstadt, Germany) solid phase Fmoc-HBTU/tert-butyl protection chemistry as previously described [13]. After synthesis was complete, the resin was divided. Half of the sample was deprotected and cleaved from the resin for use in the serum degradation and T cell activation assays, and the rest half was labeled with fluorescein before final deprotection and cleavage for use in the MHC II binding assay. Peptides were cleaved from the resin using 90% trifluoroacetic acid (Sigma-Aldrich Corp. St. Louis, MO), 4% tetraiodosilane (Sigma) and 6% water for 3 hours and then precipitated with diethyl ether (Sigma). The solvent was decanted and dried for 24 hrs under high vacuum. The peptides were then dissolved in dimethylsulfoxide (DMSO) and purified by reverse phase high performance liquid chromatography (RP-HPLC) on an acetonitrile/water gradient (Waters, Milford, MA). The peptides were characterized by electrospray ionization mass spectrometry (ES-MS) on a LCQ Advantage Max mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Aza-peptides were synthesized following the same methodology with the exception of aza-glycine insertion by chemical synthesis at the desired substitution

points during elongation as described previously [13]. Some of the sequences that were synthesized are represented in Table 1.

MHC II Binding Assay

Various MBP Ac1–9 peptides were labeled with fluorescein for use in MHC II binding studies. Soluble I-A^u MHC II protein was isolated from BW5134 cell lysate (National Cell Culture, Minneapolis, MN) and purified by M115 antibody exchange column (Millipore, Billerica, MA). Isolated protein was stored at 4°C until utilized in the assay. An excess of APL (5 µM) was incubated with the isolated MHC II protein (50–100 µl) overnight at 37°C. The excessive unbound peptide was then removed by Sephadex G50-SF (Sigma) spin column (~1 ml) purification so that only the peptide/protein complex remained. The complex was then incubated at 37°C and samples (50 µl) were taken at time points determined as the experiment progressed. Sample composition was monitored by HPLC (Shimadzu, Columbia, MD) on a size exclusion column (Phenomenex, Torrance, CA). The retention time of the peptide/MHC II complex was different than the labeled free peptide which allows for semi-quantification of the dissociation. The peak height for the complex is graphed versus time and the resulting curve is used to calculate the disassociation half time of labeled MBP peptide from MHC II protein.

Serum Degradation Half Life Assay

The unlabeled APLs were tested to determine their resistance to protease degradation. The serum degradation experiments are a modification of published methods [17] utilizing matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The peptides were dissolved at 10mM in sterile H₂O and then 30 µl was added to 270 µl of rat serum (Sigma). A degradation resistant peptide composed of D-amino acids was used as an internal standard and was added to the serum at the same concentration. The mixture was then incubated at 37°C and 20 µl samples were taken at specified time points. The time points vary with the degree of protease resistance of the peptide and the optimum times in order to calculate a half-life were determined during testing. The 20 µl sample was added to 80 µl of a 3:1 methanol:ethanol mixture to precipitate the proteins from the serum and then spun in a microcentrifuge (Eppendorf, Westbury, NY) at 12,000 g for 10 minutes. Supernatant, which contained the peptide, was then combined with α-cyano-4-hydroxycinnamic acid matrix and spotted on a gold MALDI plate (Applied Biosciences, Foster City, CA) in triplicate. The ratio between the internal standard peptide peak and the test peptide peak was then determined using a Voyager-DE STR Biospectrometry Workstation (Applied Biosciences) by MALDI-TOF MS. The ratio was graphed against the sample times. The degradation half-life was then determined from the equation obtained from the natural log of the ratio graphed against sample times using Microsoft Excel (Microsoft, Bellevue, WA).

T cell Activation Assay

The T cell activation experiment is a multi-cell *in vitro* test of the biological potential resultant from modification of the native peptide sequence. The assay utilized L929 cells as an antigen presenting cell (APC). These cells have been transfected with I-A^u MHC II protein [15] specific to MBP Ac1–11 and present antigen to the 172.10 T cell hybridoma

[16]. The 172.10 cells express a TCR that specifically recognizes MBP Ac1–11 peptide in the context of I-A^u MHC II molecules. The 172.10 T cell hybridomas produce the cytokine IL-2 if they are activated through sufficient recognition of their antigenic peptide. IL-2 production by the 172.10 cells was used as an indication of the degree of stimulation through the TCR in response to the APL being tested. Aza-peptides from the binding experiment as well as unlabeled APLs with and without aza-amino acid substitutions were tested. Serial dilution of test peptides were added to 3×10^4 L929 cells and 6.5×10^4 172.10 cells in 96 well plates (Corning, Corning, NY) and incubated at 37°C for 48 hrs. Supernatant samples were then taken and frozen at –80°C until the cytokine analysis was performed. The IL-2 concentration was determined by sandwich ELISA (BD Bioscience, San Diego, CA) in a 96 well plate format following standard protocols. The absorbance of the ELISA plates was read with a Vmax absorbance plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Samples were quantified by comparing absorbance readings to a standard curve prepared with known concentrations of IL-2 following the kit directions.

EAE Induction and Treatment

Male B10.PL-H2uH2-T18a/(73NS)SnJ mice that were 3–5 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). EAE was induced when mice were in the mass range of 18–20 grams. To induce EAE, mice were immunized with (1:1) CFA containing *Mycobacterium tuberculosis* H37Ra (10 mg/ml; Difco) and phosphate-buffered saline (PBS) containing guinea pig MBP (1 mg/ml). Each mouse received 4 s.c. injections, one over each haunch, totaling 400 µl of an emulsion containing 400 µg of MBP. Control animals received PBS/CFA alone. At the time of the induction the mice also receive an i.p. injection of pertussis toxin (Sigma) (200 ng/mouse) and again 48 hours later. After induction of EAE, mice were monitored daily for weight changes and analyzed for clinical signs. The clinical scores for severity of EAE signs were based on the following grades: 0-no change, 1-limp tail or waddling gait with tail tonic, 2-partial hind-limb paralysis, 3-hind-limb paralysis, 4-hind-limb paralysis with front-limb weakness or partial paralysis, 5-quadruplegic or moribund. Half number scores were used if the animal was partway between clinical score criteria levels.

Treatment: The APL was dissolved to a concentration of 1.25 mM. The mice received 200 µl of the APL solution resulting in a dose of either 250 nmol. The dose was given in 200 µl i.p. every other day starting on day 7 and continuing through day 21 for a total of 8 doses following the protocol established by a previous study [18].

Analysis of T Cell Transcription Factors and Cytokine Genes by RT-PCR

Total RNA was extracted from splenocytes using the RNEasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. The reverse transcription reaction was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Briefly, the following components were combined to form a 20 µL reaction volume: nuclease-free water, 5× iScript Select reaction mix, oligo (dT)20 primer, total RNA (2 µg), and Reverse Transcriptase (RT).

The reaction tubes were first incubated for 90 minutes at 42°C, and then incubated for 5 minutes at 85°C (to inactivate RT). PCR was executed in a programmed thermal cycler (Biometra, Germany). β -actin mRNA was used as a control for each experiment using the primer sequence 5'-ATATCGCTGCGCTGGTCGTCGA-3' (sense) and 5'-ACCCATTCCCACCATCACACCCTG-3' (anti-sense). Cytokine and transcription factor primers were used to test for FoxP3 5'-TCCTTCCCAGAGTTCTTCCA-3' (sense) 5'-GGCTAGGTTGGAAGTAGGGG 3'(anti-sense); IL-17 5'-CTCCAGAAGGCCCTCAGACTA-3' (sense) and 5'-AGCTTTCCCTCCGCATTGACACAG-3' (anti-sense); Tbet 5'-CACCGGAGCCCACTGGATGC -3' (sense) and 5'-TGCAGGACGATCATCTGGGTCACA-3' (anti-sense); STAT4 5'-CTCCCAGCCGTGCGAAGTCTC-3' (sense) and 5'-GGCTCCGTGGAATCGCTTCGG-3' (anti-sense); Thermal cycling parameters were 95°C for 10 minutes followed by 40 cycles of amplifications at 96°C for 3 seconds, 55°C for 3 seconds, and 68°C for 5 seconds, followed by a final elongation step of 72°C for 10 minutes. PCR products were visualized using Geldoc software following electrophoresis in 1.5% agarose gel with ethidium bromide staining.

Results

TCR/MHC II Complex Leads to Design of MBP Aza-Peptides

The use of APL as a treatment in humans remains a worthwhile therapeutic to investigate because of its selective target rather than broad immunosuppression commonly seen in known drugs on the market for MS and other autoimmune diseases. However, bioavailability optimization is needed to reduce hypersensitivity reactions induced by currently available APLs.

The TCR/MHC II complex is specific for the antigens that it will bind, but some degree of promiscuity binding is demonstrated with regard to the peptide sequence that will interact within the binding groove [19]. Once the TCR and MHC II contact points have been identified, it is possible to perform logical substitutions to the native peptide sequence to alter the properties of the ligand in order to either increase or decrease binding [20]. A molecular model of the MBP Ac1-9 peptide binding to the I-Au MHC II was used, which helped determine where the residue/peptide contacts the TCR and MHC II (Figure 1A) [21]. The contact points of the MBP Ac1-9 peptide to the I-Au MHC II molecule and 172.10 TCR were subsequently confirmed by the crystal structure [22]. Since structural modifications can alter the biokinetic profile of a protein - including solubility and protease resistance - aza-amino acid substitutions to the APLs (Figure 1B) were used. These types of substitutions are known to have MHC II binding, T-cell activation potency equal to the native peptide, but most importantly aza-peptides have shown resistance to amino- and carboxypeptidases.

Several candidate aza-APLs were designed based on the Ac1-9 native MBP sequence. The type of amino acids that interact with the MHC II molecule and are available to the TCR (Figure 1A) can be determined from the crystal structure. Previous published work suggested that aza-glycine substitutions near a TCR contact point produce T cell partial

agonist that may have therapeutic potential [13]. Nonetheless, stronger binding to the MHC II is promoted by tyrosine, and this too was taken into account during designing of the APLs. The main TCR contact points of interest are the glutamine (Q) in the 3rd position and the proline (P) in the 6th position. Placing an alteration at or next to these points could cause significant favorable changes. As seen in Table 1, five candidate aza-APLs and several controls were created. Controls were the native MBP sequence, a null sequence that does not have proper contact to the TCR and thus produces no binding, a strong agonist in which the MHC II contact point has been changed from lysine to alanine (K→A), and a known agonist where the MBP contact point has been changed from lysine to threonine (K→T).

The candidate aza-APLs were then created logically by changing near or at TCR contact points (secondary and primary contact points respectively). 2azaG4A changes the native sequence to having an aza-glycine at position 2 and an alanine at position 4; the aza-glycine for TCR contact changes and protease resistance and the alanine for stronger MHC II binding. 3azaG4Y changes the native sequence to having an aza-glycine at position 3 and a tyrosine at position 4; the aza-glycine for TCR contact changes and protease resistance and the tyrosine for stronger MHC II binding. 4Y5azaG changes the native sequence to having an aza-glycine at position 5 and a tyrosine at position 4; the aza-glycine for TCR contact changes and protease resistance and the tyrosine for stronger MHC II binding. The candidate 4F changes the native sequence at position 4 from a lysine to having a phenylalanine for stronger MHC II binding. Lastly, 2azaGF changes the native sequence to having an aza-glycine at position 2 and a phenylalanine at position 4; the aza-glycine for TCR contact changes and protease resistance and the tyrosine for stronger MHC II binding.

APL candidates and MHC II binding

In order for a CD4+ T cell to respond to an antigen and for activation to take place, the TCR must interact with an antigen/MHC II molecule complex present on the surface of an APC [23]. The TCR will interact with the antigen/MHC molecule even if there is some variation in the antigenic peptide's sequence allowing for a greater immunological range [3]. Changes to this sequence due to amino acid substitution can increase or decrease the binding time and vary the resulting response from the T cell. The candidate APLs were tested for binding to soluble I-Au MHC II to determine if the modification made maintained the peptide/MHC II interaction in order to predetermine potential usefulness in a cellular system. A graphical representation of the calculated dissociation half times from I-Au MHC II molecule of the various labeled APLs shows differential binding abilities (Figure 2).

Some substitutions resulted in stronger binding with increased half off binding times (3azaG4Y, 4YazaG) and some substitutions resulted in shorter binding times (2azaG4A) that were comparable to the native 4K MBP Ac1-9 peptide sequence. The aza-peptide substitutions altered the binding kinetics but the binding still occurred, resulting in dissociation half off times that ranged between the native MBP Ac1-9 peptide (4K) (15 minutes) and a known strong binding (4Y) (3,222 minutes) sequence. 3azaG4Y and 4YazaG remain candidates based on increased MHC II binding.

Increased Protease Resistance of Aza-APLs

The degradation times of the peptides in rat serum were determined to assess if aza-amino acid substitution provided improved protease resistance. Rat serum was utilized because the native MBP sequence is of rat origin. The ratio of an APL to a resistant internal standard at various time points following incubation in rat serum, as described, were analyzed and graphed as a curve. The derived formula for the curve is used to calculate the degradation half-life of the test peptide. The calculated degradation half-life of the native MBP Ac1–9 peptide is compared to the degradation half-life of other natural amino acid APLs as well as to aza-APLs in Figure 3.

Comparing the natural peptide APLs to the native sequence, the substitution of alanine at position 4 did not appreciably alter the half-life (6% difference). Alternatively, a phenylalanine substitution at position 4 (4F) markedly increased the half-life (48% increase). A tyrosine substitution at position 4 (4Y) markedly decreased the half-life (69% decrease) compared to the native lysine (4K) at position 4. Substitution with aza-glycine at position 2 or 3 produced APLs that had longer half-lives than the native sequence. These aza-APLs also possessed half-lives that were longer than their corresponding native peptide APL. In fact, insertion of an aza-glycine at position 3 resulted in an APL that was completely resistant to degradation in rat serum. The substitution of aza-glycine at position 5 conversely results in an APL with a half-life that was less than the native sequence (81% less) and also less than the native 4Y APL (38% less). Compared to the native MBP sequence, 2azaG4F and 3azaG4Y showed the best resistance to degradation in rat serum.

Effect on T cell Activation

T cell activation assays were performed to assess if the changes made to the MBP sequence preserved their potential bioactivity. In general, strong agonists produce a robust IL-2 response whereas partial agonists will produce weak to no IL-2 response. The current study demonstrated that modest changes to the MBP native sequence create variable changes to T cell activation through the TCR that recognizes MBP Ac1–11 (Table 2). The bioactivity of these APLs with aza-amino acid substitutions is demonstrated in a cellular system by utilizing cytokine production as an end point. Table 2 displays relative IL-2 responses for some of the peptides that were tested for IL-2 production by the 172.10 T cell hybridomas when stimulated with MBP APLs. From these results, 4A was confirmed as a strong agonist as well as the native sequence, 4F and 2azaG4F. Partial agonists and candidates for *in vivo* treatment would be 2azaG4A, 3azaG4Y and 4Y5azaG. Weak or partial agonism due to a small IL-2 response is seen with 2azaG4A, 3azaG4Y, and 4Y5azaG. In general, weak or partial agonists are preferred because they do not cause T cell proliferation as compared to agonists [5].

Aza-APL attenuates disease in EAE

Based on all of the screening stated above, 3azaG4Y (3aza) APL was selected as a best candidate and tested its efficacy in mouse model of EAE. Data obtained suggest that 3aza showed increased MHC II binding compared to the native sequence, as well as protease resistance in rat serum and partial agonist activity in the IL-2 study. All other candidates met only 2 of the 3 requirements or less.

To study functionality *in vivo*, B10.PL mice were used to induce EAE and treated with 3aza intraperitoneally. Not only was the aza-APL found to ameliorate the disease significantly, disease onset was also delayed as compared to untreated controls (Figure 4A). Examination of the inflammatory and anti-inflammatory T cell markers showed a reduction of inflammation (Figure 4, B&C). More specifically, IL-17 cytokine gene expression which is a marker for Th17 inflammatory cells was reduced (Figure 4B). Anti-inflammatory T regulatory cell marker FoxP3 gene was also increased with 3aza treatment. Markers for inflammatory Th1 cells such as STAT1 and T-bet genes were also reduced (Fig. 4C).

Discussion

Utilization of aza-peptide substituted APLs provides a novel solution to the problem of *in vivo* hypersensitivity response to peptide-based drugs through increased protease resistance of the peptide sequence thereby decreasing the required therapeutic dose. This study demonstrated the possibility of making subtle changes to the native antigenic sequence while maintaining binding to an MHC II presentation molecule. Maintaining the binding to MHC II molecules is the first check point to any changes that are made to the peptide sequence. If the changes are so great that binding is no longer possible then the peptide will not be properly presented to the target T cells. The data in Figure 2 demonstrates that aza-peptide insertion affects the binding to the MHC II molecule but a minimum binding is still preserved. Even if the aza-peptides themselves would significantly reduce the binding, rational changes to the APL sequence, such as position four substitutions, can be performed to create scaffold sequences with greatly increased binding.

These scaffolds can then force the MHC II molecule to present an APL with an aza-glycine substitution. Overly strong binding is not the only goal of APLs though as variable binding strengths lead to altered T cell responses which are difficult to predict based solely on the sequence modifications [13]. Modification of the MHC II anchors has been demonstrated previously as an effective method for creation of partial agonist APLs [24]. Therefore, no matter what logical substitutions are made to the sequence based on the contact points (Figure 1), testing is still required.

Increased resistance to protease degradation by altering the native MBP antigenic sequence with aza-amino acids was also demonstrated. The utilization of APLs and peptides as drugs has the potential side effects of hypersensitization and allergic reactions [8, 9]. The previously tested APLs that made it to clinical trials were stopped as a result of unintended allergic response potentially due to the high concentrations required. These high concentrations were required due to low bioavailability as well as inadequate optimization. There are various modifications to a peptide sequence which produce protease resistance. N-terminal acetylation increases protease resistance compared to an unacetylated terminus (data not shown). The native sequence of MBP Ac1–9 is N-terminally acetylated, so our natural peptide already has a higher protease resistance capacity compared to a peptide sequence with a non-acetylated N-terminus. The strategy of using aza-peptides will potentially reduce the concentration required for clinical effectiveness of APLs through further increased protease resistance. The modified aza-peptides do not occur naturally so proteases will not have the evolutionary exposure to develop an active site that can cleave

these (peptide) bonds. These aza-peptide substitutions produced a further decrease of protease degradation (Figure 3) compared to the native (4K) sequence. The aza-amino acid substitutions were synthesized with aza-glycine, which resulted in increased protease resistance (4A compared to 2azaG4A, and 4F compared to 2azaG4F). Multiple aza-amino acid substitutions or aza-amino acid substitutions beside glycine are possible and may result in even greater resistance and deserve further study.

TCR activation by the peptide still remains to be tested. Hyperactivation of T cells with strong agonists are not suitable for the induction of therapeutic responses. In this study, an aza-APL that would be classified as a partial agonist and met all of the previous criteria was synthesized and screened. *In vivo* studies with the aza-APL proved to be sufficient in reducing disease severity and inflammation in EAE mice. The clinical signs as well as disease onsets were significantly reduced when 3aza was administered in EAE mice. These data provide significant evidence for this type of chemical modification and aza-APL synthesis being applicable for drug design. Furthermore, analysis of peripheral inflammation showed a significant reduction of inflammatory immune markers in treated animals as compared to untreated controls. Reduction of both Th17 and Th1 cell type markers was also complemented with an increase in Treg cell type markers, confirming the T cell specific route is affected by 3aza.

Aza-peptide treatment significantly decreased Stat4 and IL-17, suggesting the importance of this modified peptide in diminishing the effector functions of Th1 and Th17 cells in the host and attenuation of EAE. STAT4 has also been shown to regulate GM-CSF production by CD4⁺ T cells and this cytokine is necessary for the induction of EAE [25], thus inhibition of Stat4 by 3aza may prove important in designing novel therapeutics against EAE/MS. Studies are underway to determine whether aza peptide perturbs a host of cytokines/chemokines and modulates pathogenic T cell responses and the disease in EAE mouse model of MS. Aza-peptide inclusion in APLs is a viable technique for the creation of immune modulatory peptides that display properties of the native sequence yet possess increased resistance to protease cleavage. Here, the use of aza-APLs that mimic MBP was demonstrated as useful in treating EAE, but this same concept would be useful in other autoimmune diseases that involve specific antigenic sequences. In summary, aza-APLs could be applied to any *in vivo* situation where peptide based therapies are relevant and provides a potential strategy for decreasing the hypersensitivity side effects that have, to this point, plagued many of the clinical trials and applications as therapeutic treatments.

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References

1. Dustin ML. T cell activation through immunological synapses and kinapses. *Immunol Rev.* 2008; 221:77–89. DOI: 10.1111/j.1600-065X.2008.00589.x [PubMed: 18275476]

2. Karin N, Mitchell DJ, Brocke S, Ling N, Steinman L. Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon gamma and tumor necrosis factor alpha production. *J Exp Med.* 1994; 180:2227–37. [PubMed: 7525850]
3. Hemmer B, Vergelli M, Gran B, Ling N, Conlon P, Pinilla C, Houghten R, McFarland HF, Martin R. Predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *J Immunol.* 1998; 160:3631–6. [PubMed: 9558061]
4. Correale J, Farez M, Gilmore W. Vaccines for Multiple Sclerosis: Progress to Date. *CNS Drugs.* 2008; 22:175–198. DOI: 10.2165/00023210-200822030-00001 [PubMed: 18278975]
5. Rabinowitz JD, Beeson C, Wulfinf C, Tate K, Allen PM, Davis MM, McConnell HM. Altered T cell receptor ligands trigger a subset of early T cell signals. *Immunity.* 1996; 5:125–135. doi: [http://dx.doi.org/10.1016/S1074-7613\(00\)80489-6](http://dx.doi.org/10.1016/S1074-7613(00)80489-6). [PubMed: 8769476]
6. Mazzanti B, Hemmer B, Traggiai E, Ballerini C, McFarland HF, Massacesi L, Martin R, Vergelli M. Decrypting the spectrum of antigen-specific T-cell responses: the avidity repertoire of MBP-specific T-cells. *J Neurosci Res.* 2000; 59:86–93. [PubMed: 10658188]
7. Lutterotti A, Sospedra M, Martin R. Antigen-specific therapies in MS - Current concepts and novel approaches. *J Neurol Sci.* 2008; 274:18–22. DOI: 10.1016/j.jns.2008.05.021 [PubMed: 18599087]
8. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, Gran B, Eaton J, Antel J, Frank JA, McFarland HF, Martin R. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med.* 2000; 6:1167–75. DOI: 10.1038/80516 [PubMed: 11017150]
9. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, Steinman L. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med.* 2000; 6:1176–82. DOI: 10.1038/80525 [PubMed: 11017151]
10. Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Annals of Neurology.* 2006; 60:12–21. DOI: 10.1002/ana.20913 [PubMed: 16802293]
11. Boonen K, Creemers JW, Schoofs L. Bioactive peptides, networks and systems biology. *Bioessays.* 2009; 31:300–14. DOI: 10.1002/bies.200800055 [PubMed: 19260025]
12. Zaliauskiene L, Kang S, Sparks K, Zinn KR, Schwiebert LM, Weaver CT, Collawn JF. Enhancement of MHC class II-restricted responses by receptor-mediated uptake of peptide antigens. *J Immunol.* 2002; 169:2337–45. [PubMed: 12193699]
13. Hart M, Beeson C. Utility of azapeptides as major histocompatibility complex class II protein ligands for T-cell activation. *J Med Chem.* 2001; 44:3700–9. [PubMed: 11606135]
14. Wipf P, Li W, Adeyeye CM, Rusnak JM, Lazo JS. Synthesis of chemoreversible prodrugs of ara-C with variable time-release profiles. Biological evaluation of their apoptotic activity. *Bioorg Med Chem.* 1996; 4:1585–96. [PubMed: 8931928]
15. Tate KM, Lee C, Edelman S, Carswell-Crumpton C, Liblau R, Jones PP. Interactions among polymorphic and conserved residues in MHC class II proteins affect MHC-peptide conformation and T cell recognition. *Int Immunol.* 1995; 7:747–61. [PubMed: 7547702]
16. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell.* 1993; 72:551–60. [PubMed: 7679952]
17. Kokko KP, Dix TA. Monitoring neurotensin[8–13] degradation in human and rat serum utilizing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Biochem.* 2002; 308:34–41. [PubMed: 12234461]
18. Leadbetter EA, Bourque CR, Devaux B, Olson CD, Sunshine GH, Hirani S, Wallner BP, Smilek DE, Happ MP. Experimental autoimmune encephalomyelitis induced with a combination of myelin basic protein and myelin oligodendrocyte glycoprotein is ameliorated by administration of a single myelin basic protein peptide. *J Immunol.* 1998; 161:504–12. [PubMed: 9647262]
19. Smilek DE, Wraith DC, Hodgkinson S, Dwivedy S, Steinman L, McDevitt HO. A single amino acid change in a myelin basic protein peptide confers the capacity to prevent rather than induce

- experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA*. 1991; 88:9633–9637. DOI: 10.1073/pnas.88.21.9633 [PubMed: 1719536]
20. Matsui K, Boniface JJ, Steffner P, Reay PA, Davis MM. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci U S A*. 1994; 91:12862–6. [PubMed: 7809136]
21. Lee C, Liang MN, Tate KM, Rabinowitz JD, Beeson C, Jones PP, McConnell HM. Evidence that the autoimmune antigen myelin basic protein (MBP) Ac1–9 binds towards one end of the major histocompatibility complex (MHC) cleft. *J Exp Med*. 1998; 187:1505–16. [PubMed: 9565642]
22. He XL, Radu C, Sidney J, Sette A, Ward ES, Garcia KC. Structural snapshot of aberrant antigen presentation linked to autoimmunity: the immunodominant epitope of MBP complexed with I-Au. *Immunity*. 2002; 17:83–94. [PubMed: 12150894]
23. Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. Stat6 is necessary and sufficient for IL-4's Role in Th2 Differentiation and Cell Expansion. *Journal of Immunology*. 2001; 166:7276–7281. DOI: 10.4049/jimmunol.166.12.7276
24. Ryan KR, McNeil LK, Dao C, Jensen PE, Evavold BD. Modification of peptide interaction with MHC creates TCR partial agonists. *Cell Immunol*. 2004; 227:70–8. DOI: 10.1016/j.cellimm.2004.01.003 [PubMed: 15051516]
25. McWilliams IL, Rajbhandari R, Nozell S, Benveniste E, Harrington LE. STAT4 controls GM-CSF production by both Th1 and Th17 cells during EAE. *J Neuroinflammation*. 2015; 12:128.doi: 10.1186/s12974-015-0351-3 [PubMed: 26123499]

Figure 1.

Peptide in Binding Cleft and Aza-amino Acid. (A) APL in binding groove between MHCII and TCR to show the contact points of each molecule and demonstrate where modifications can be made. (B) The substitution of the α -carbon in an amino acid for a nitrogen results in an aza-peptide as described by Hart and Beeson [13]. These aza-amino acids were chemically inserted into peptide sequence during synthesis of the APLs.

Figure 2.

The Half Off Time of Modified MBP from the I-Au Soluble MHC II Protein. The calculated half off times of several aza-APLs compared to the native MBP Ac 1–9 (4K) peptide from the I-Au soluble MHC II protein. The half off time in minutes is indicated above the bar.

Figure 3.

The Degradation Times of Various APLs in Rat Serum. The calculated half-lives of various substituted APL compared to the native MBP sequence (4K).

Figure 4.

Decreased Disease EAE in Mice Treated with 3aza. (A) Mean clinical score of the 3aza (200 nmol/mouse) treated group compared to the control and EAE untreated groups. Each time point represents the mean clinical score, $n = 5$. (B) Splenocyte mRNA levels of FoxP3, IL-17, STAT4 and Tbet from animals Control, EAE untreated and EAE treated with 3aza, $n=3$.

Table 1**A summary table of the aza-substitutions performed to the native MBP sequence**

The arrows above the native sequence designate the contact points where down arrows correspond to MHC II molecule contacts and up arrows correspond to TCR contacts.

Name	MBP Peptide Sequence
MBP Ac1–9/4/Native sequence	Ac-A $\downarrow$ S $\uparrow$ Q $\downarrow$ K $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
MBP Ac1–9/5k/Null sequence	Ac-A $\downarrow$ S $\uparrow$ Q $\downarrow$ K $\uparrow$ K $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
MBP Ac1–9/4A/Strong agonist	Ac-A $\downarrow$ S $\uparrow$ Q $\downarrow$ A $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
2azaG4A	Ac-A $\downarrow$ S $\uparrow$ G _{aza} $\downarrow$ Q $\uparrow$ A $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
MBP Ac1–9/4Y/Agonist	Ac-A $\downarrow$ S $\uparrow$ Q $\downarrow$ Y $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
3azaG4Y	Ac-A $\downarrow$ S $\uparrow$ G _{aza} $\downarrow$ Y $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
4Y5azaG	Ac-A $\downarrow$ S $\uparrow$ Q $\downarrow$ Y $\uparrow$ G _{aza} $\downarrow$ P $\uparrow$ S $\uparrow$ Q R
4F	Ac-A $\downarrow$ S $\uparrow$ Q $\downarrow$ F $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R
2azaG4F	Ac-A $\downarrow$ G _{aza} $\uparrow$ Q $\downarrow$ F $\uparrow$ R $\uparrow$ P $\downarrow$ S $\uparrow$ Q R

Table 2**Agonism in Aza-peptides**

The IL-2 response indicative of agonism for the various APLs relative to the native sequence.

Name (aza-substitution)	IL-2 Response
4K	++
5K	–
4A	+++
2azaG4A	+
4F	+++
2azaG4F	++
4Y	++
3azaG4Y	–
4Y5azaG	–

+++ and ++ indicates strong agonist, + and – indicates partial agonism as given by IL-2 response.