Immunomodulatory effects of a rationally designed peptide mimetic of human IFN β in EAE model of multiple sclerosis

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Abstract

The efficiency of interferon beta (IFN β)-based drugs is considerably limited due to their undesirable properties, especially high immunogenicity. In this study, for the first time we investigated the impact of a computationally designed peptide mimetic of IFN β , called MSPEP27, in the animal model of MS.

A peptide library was constructed using the Rosetta program based on the predominant IFNAR1-binding site of IFN β . Molecular docking studies were carried out using ClusPro and HADDOCK tools. The GROMACS package was subsequently used for molecular dynamics (MD) simulations. Validation of peptide-receptor interaction was carried out using intrinsic fluorescence measurements. To explore *in silico* findings further, experimental autoimmune encephalomyelitis (EAE) was induced by subcutaneous immunization of myelin oligodendrocyte glycoprotein (MOG35-55). Mice were then separated into distinct groups and intravenously received 10 or 20 mg kg⁻¹ of MSPEP27 or IFN β . The inflammatory mediators were monitored by immunohistochemistry (IL17, CD11b, CD45), quantitative real-time PCR (MMP2, MMP9, TIMP-1) and enzyme-linked immunosorbent assay (IL1 β , TNF α) methods.

Among the library of tolerated peptides, MSPEP27, a peptide with favorable physicochemical properties, was chosen for further experiments. This peptide was shown to significantly interact with IFNAR1 in a dose-dependent manner. Like IFN β , MSPEP27 could efficiently bind to IFNAR1 and form a stable peptide-receptor complex during 30 ns MD simulations. *In vivo* analyses revealed that MSPEP27 could lessen inflammation by modulating the levels of inflammatory mediators.

According to our results, MSPEP27 peptide could efficiently bind to IFNAR1 and suppress neuroinflammation *in vivo*. We conclude that MSPEP27 has protective effects against MOG-induced EAE via reduction of immune dysfunction and inflammation.

Key Words: Multiple sclerosis, IFN β , peptide, MSPEP27, EAE, inflammation

Abbreviations list

MS: Multiple sclerosis

CNS: Central nervous system

IFN β : Interferon beta

IFNAR1: Type I IFN receptor

RRMS: Relapsing–remitting multiple sclerosis

EAE: Experimental autoimmune encephalomyelitis

i.v: Intravenously

ICVI: Intracerebroventricular

BBB: Blood–brain barrier

3D: Three dimensional

PDB: Protein Data Bank

GRAVY: Grand average of hydropathicity

MD: Molecular dynamics

RMSD and Rg: Root mean square deviation and radius of gyration

ns: Nanosecond

ps: Picosecond

VdW: van der Waals

IL-17: Interleukin-17

CD11b: Human leukocyte adhesion glycoprotein Mac-1 α

TIMP-1: tissue inhibitor of matrix metalloproteinase 1

MMP2/9: Matrix metalloproteinase 2/9

IL1 β : interleukin 1 β

TNF α : Tumor necrosis factor-alpha

1. Introduction

Multiple sclerosis (MS) is a highly varied and unpredictable autoimmune disease which is speculated to attack the myelin proteins in the central nervous system (CNS). One of the most important hallmarks of the MS process is that peripherally activated inflammatory cells can pass across the blood-brain barrier (BBB) leading to demyelination within the CNS [1]. Numerous studies have been undertaken to understand the key inflammatory mediators and neurodegenerative mechanisms that underlie the relapsing, and eventually progressive phase of the disease. Consequently, there are several natural- and synthetic-based treatment strategies which are widely used to alleviate the exacerbations of MS [2, 3]. Several interferon beta (IFN β)-based formulations such as Avonex, Betaseron, Plegridy and Rebif have been shown to attenuate the MS signs and are used as first-line treatment for relapsing–remitting multiple sclerosis (RRMS) [4]. However, IFN β therapy is substantially associated with a number of adverse side effects including flu-like symptoms, inconsistencies in patient laboratory analyses, menstrual disorders, and increased spasticity [5]. Moreover, similar to other clinically available protein-based drugs, IFN β has a range of drawbacks including the high number of antigenic regions, its high cost and susceptibility to proteolytic degeneration [6, 7]. Peptides can be assumed as suitable alternatives of IFN β for treatment of MS. These compounds have several unique physicochemical properties that make them attractive in medical interventions [8, 9].

In a previous study, Sato and colleagues isolated a 15-mer peptide (SVQARWEAAFDLDLY) mimetic of IFN β , named SYR6, using phage-display screening which could efficiently bind to type I IFN receptor (IFNAR1) in a concentration-dependent manner [10]. This peptide could effectively elicit antiviral activity *in vitro*, even in the presence of neutralizing monoclonal antibodies against interferon types. However, the authors reported that the deduced amino acid

sequence of SYR6 had no similarity to the primary sequence of IFN β . In a separate study, the structural basis of IFN β -IFNAR1 interactions was studied using crystallography [11]. Interferons are known to initiate specific signals via formation of the heterodimeric IFNAR1-IFNAR2 complex. However, the authors of this study found that IFN β was able to induce intracellular signals through binding with IFNAR1, independent of IFNAR2. Thus, IFN β can be considered as a promising candidate for designing mimetic peptides in MS therapy because there is no apparent need to target both IFNAR1 and IFNAR2, simultaneously. Here, we have rationally designed and improved a peptide mimetic of IFN β , designated MSPEP27, based on the evolutionary, structural and dynamic information of the IFN β -IFNAR1 complex as well as literature-based data. The peptide was capable of significantly reducing brain dysfunction in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS by down-regulating the production of inflammatory mediators.

2. Methods

2.1. Rational design of mimetic peptide *in silico*

2.1.1. Evolutionary and functional studies

In order to identify conserved residues in the primary sequence of IFN β , a multiple sequence alignment was performed using the default parameters of the Clustal W program [12]. First, the amino acid sequences of IFN β in human, mouse and rat were obtained from UniProt databank (<http://www.uniprot.org/>). Subsequently, MSA was performed and the aligned sequences were visualized using the Jalview program [13]. In parallel, the ConSurf server was used to predict the putative functional residues involved in IFN β activity [14]. This algorithm identifies the patches

of highly conserved (or occasionally variable) residues of a given three dimensional (3D) structure which are generally associated with an important biological function.

2.1.2. Preparation of 3D structures of IFN β and IFNAR1

The 3D structure of human IFN β (PDB ID: 1AU1, Resolution: 2.2 Å) and IFNAR1 (PDB ID: 3S98, Resolution: 1.9 Å) were obtained from Protein Data Bank (PDB) (<http://www.rcsb.org/pdb/home/home.do>). To gain insights into the IFN β -IFNAR1 mode of interaction, the NMR structure of murine Ifn β in complex with Ifnar1 was downloaded from PDB (PDB ID: 3WCY) databank. For structural alignment analyses, the unbound structure of murine Ifn β (PDB ID: 1WU3, Resolution: 2.15 Å) was also provided.

2.1.3. Structural alignment of IFN β /Ifn β and IFNAR1/Ifnar1

After evaluating the mode of interaction for the murine Ifn β -Ifnar1 complex, a structural alignment was applied to identify the structurally conserved residues between the human and murine proteins. The unbound structures of human IFN β and murine Ifn β were structurally aligned using SuperPose and TM-align servers [15, 16]. A structural alignment was also carried out between human IFNAR1 and murine Ifnar1 utilizing the tools above. However, the unbound structure of murine Ifnar1 was not available in PDB databank, and therefore the 3WCY pdb file was retrieved using PyMOL software [17].

2.1.4. Molecular docking studies

Molecular docking studies were applied utilizing two popular web tools, ClusPro and HADDOCK [18, 19]. The ligand-binding site of human IFNAR1/2 was determined according to the study of Chill and colleagues [20]. The IFNAR-binding site of human IFN β was also

predicted according to the receptor-binding site of murine Ifnar1. Consequently, both blind and high-resolution docking runs were separately performed. Finally, the protein complexes with lowest binding energy were chosen and visualized using LigPlot+ software to understand the functionally interacting residues of IFN β and IFNAR1 [21]. Prior to docking, 3D structures of the peptides were predicted using the PEP-FOLD server [22]. The solution structure of SYR6 peptide was obtained from PDB database (PDB ID: 1ID7).

2.1.5. Peptide library construction and improvement of physicochemical properties

The physicochemical properties of the initial peptide (MLQNIFAIFRQDSSSTGWNENITIVENLL) were improved via several possible amino acid substitutions. A peptide library was then constructed using Rosetta backrub with sequence tolerance protocols implemented in the Rosetta3.5 software and the most tolerated peptide sequence was determined among hundreds of generated peptides [23]. It should be noted that critical functional residues involved in interaction with IFNAR1 were not altered. Physicochemical features including molecular weight, theoretical pI, net charge at pH 7, instability index, grand average of hydropathicity (GRAVY), number of aggregation hot spots, number of antigenic regions, half-life, water solubility and cell-penetrating capability were calculated using ProtParam (<http://web.expasy.org/protparam/>), peptide property calculator of Innovagen (<http://pepcalc.com/>), Aggrescan (<http://bioinf.uab.es/aap/>), IEDB (<http://tools.iedb.org/bcell/>) and CellPPD (<http://crdd.osdd.net/raghava/cellppd/>) web tools. In parallel, the improved peptides were also docked with IFNAR1 to evaluate their IFNAR1-binding affinity.

2.1.6. Molecular dynamics (MD) simulations

MD simulations were performed using GROMACS package version 5 [24]. The 3D structure of initial peptides, MSPEP27, SYR6 and IFN β in complex with IFNAR1 were solvated in a solvation box with 10.5 Å distance between the edges of the box and the protein fragments. Na⁺ and Cl⁻ ions were added into the box to neutralize the system. Subsequently, the entire systems were minimized and equilibrated for 100 ps using canonical (NVT) and the isothermal–isobaric (NPT) ensembles. Then, the systems were subjected to a 30 ns MD simulation using the leap-frog algorithm with an integration time step of 0.002 ps.

2.1.7. Energetic analyses

An average structure was prepared for each peptide-IFNAR1 and IFN β -IFNAR1 complexes from the last 3 ns MD simulations and subjected to the PRODIGY web tool for calculating the ΔG of interactions as well as determining their polar and nonpolar contacts [25]. This server calculates the number of interfacial contacts (ICs) and the percentage of non-interacting surface (NIS) in a protein-protein complex which has been shown to be crucial in physical protein-protein interactions. In parallel, the electrostatic energies were calculated using the PBEQ-Solver tool utilizing 31 snapshot configurations taken from the last 3 ns MD productions of the peptide-IFNAR1 complexes and subsequently averaging the values [8]. The van der Waals (VdW) energies were calculated using a standard GROMACS utility.

2.1.8. Intrinsic fluorescence measurements

Fluorescence measurements were performed using an RF-5000 Shimadzu spectrofluorimeter with a cell compartment thermostated at 293 K. Protein intrinsic fluorescence experiments were carried out at constant IFNAR1 concentration titrated with different MSPEP27 concentrations, to

reach a maximum molar ratio of MSPEP27-IFNAR1 equal to 10. The concentration of IFNAR1 was 3 μM and the concentrations of MSPEP27 were from 0 to 30 μM to reach the 10 molar ratio of [MSPEP27]/[IFN β]. Emission spectra were recorded from 310 to 450 nm with an excitation wavelength of 280 nm and at a scan rate of 60 nm/min. The results of all measurements of fluorescence were corrected by the results of the same titrations of medium without IFNAR1. Spectral resolution was 5 nm for both excitation and emission. In these experiments, 1.0 mL solution with a fixed concentration of MSPEP27 was accurately added into the quartz cell with a 1 cm path length and was manually titrated by successive additions of MSPEP27 at 5 min time intervals. The fluorescence emission spectra were then measured, and the maximum fluorescence intensity (about 340 nm) was used to calculate the binding constant parameters. The binding constant (K_a) and the number of binding (n) were calculated according to the following equation 2.2.8 [26]:

$$\log \frac{F_0 - F}{F} = \log K_a + n \log [P] \quad (1)$$

where F_0 and F are the fluorescence emission intensities of IFNAR1 in the absence and presence of the MSPEP27, respectively, $[P]$ is MSPEP27 concentration, K_a is the binding constant, and n is the apparent molar ratio of [MSPEP27]/[IFNAR1] complexes.

2.1.9. Visual presentations

All peptide and protein structures were depicted using PyMOL and UCSF chimera programs [27]. The graphs resulted from analysis of MD simulation trajectories were plotted using the Grace software (GRACE: <http://plasma-gate.weizmann.ac.il/Grace/>).

2.2. *In vivo* validations

2.2.1. Animals and EAE induction

Naive, C57BL/6 mice (male, 8 weeks old, 20-25 gr weight, purchased from Pasteur Institute, Karaj, Iran) were housed with a 12-hour light/dark cycle with access to water and food *ad libitum* in specific pathogen-free conditions. The animal experiments were carried out according to the protocols approved by the university of Zabol animal care and use committee. In EAE group mice were anesthetized with isoflurane and subcutaneously injected with 250 μg (100 μl) of MOG35-55 (myelin oligodendrocyte glycoprotein; Alexis Company, Lausanne, Switzerland) 1:1 suspended in 100 μl complete Freund's adjuvant (CFA) into the hind flank. Mice were also intraperitoneally (i.p.) injected with 400 ng of pertussis toxin (Difco Laboratories, Livonia, MI) in 200 μl of phosphate buffered saline (PBS). A second, identical injection of pertussis toxin was given two days following the first immunization. Injections were distributed over four spots (i.e., ~100 μl per site) across the flank areas [28].

2.2.2. Study design and experimental groups

To investigate that whether therapeutic (or post-onset) treatment with MSPEP27 could modulate the EAE, mice received intravenous (i.v.) injections of 100 μl containing different doses of IFN β and MSPEP27 (0, 10 and 20 mg kg^{-1} dissolved in 0.2 M PBS) into the right lateral ventricle of mouse on day 14 of EAE induction. One hundred twenty six mice were divided randomly into seven groups: (i) vehicle group ($n = 18$) received injection of PBS solution as vehicle at day 14 of EAE induction; (ii) sham group ($n = 18$) received pertussis toxin without MOG; (iii) EAE groups ($n = 90$) received pertussis toxin with MOG and were divided into five separate subgroups (18 mice per subgroup), (iiia) without vehicle, MSPEP27 and IFN β injection, (iiib)

with 10 mg kg⁻¹ body weight injection of MSPEP27 at day 14 of EAE induction (iiic) with 20 mg kg⁻¹ body weight injection of MSPEP27 at day 14 of EAE induction (iiid) with 10 mg kg⁻¹ body weight injection of IFN β at day 14 of EAE induction (iiie) with 20 mg kg⁻¹ body weight injection of IFN β at day 14 of EAE induction. At the end of experiments 5 animals in each group were used for immunohistochemistry (IHC), mRNA expression and protein analysis. All measurements were performed by an observer blinded to group assignments. The doses of IFN β and MSPEP27 administration were considered to be within the range of previously reported treatments [29]. As MSPEP27 is a mimetic peptide of IFN β , we used it with some modifications to better compare with IFN β . To bypass the blood–brain barrier and other mechanisms that limit systemic drug distribution into the brain, ICVI route of administration was selected for this research to allow CNS entrance of exact concentrations used for both MSPEP27 and IFN β .

2.2.3. Model characteristic and clinical evaluation

Mice were scored, blinded, as follows: 0, no overt signs of disease; 1, limp tail; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis of one (hemiparesis) or both sides (paraparesis); 4, complete hind limb paralysis of one (hemiplegia) or both sides (paraplegia); 5, moribund state. The day of immunization was considered as EAE day 0 and clinical behavior of mice was scored daily in accordance with the criteria described by Peiris et al. [30].

2.2.4. Immunohistochemical (IHC) analysis

On the 21st day of EAE induction in each of the experimental groups, animals were *i.p.* euthanized using ketamine (50 mg kg⁻¹) and xylazine (4 mg kg⁻¹), followed by cervical dislocation and opening the diaphragm. Thereafter, mice were transcardially perfused with PBS and 4% PFA (paraformaldehyde). Brains were dissected and post-fixed for 24 h in 4% PFA and

embedded in paraffin. Fixed brains were coronal sectioned (8 μ m), and the cerebral cortex was identified in accordance with the stereotaxic mouse brain atlas and used for staining and IHC analysis. Slides were incubated in blocking solution (PBS containing 2% BSA, 1% normal goat serum and 0.2% Triton X-100), and afterward incubated for 1 h in permeabilization buffer (10% goat serum, 0.1% Triton X-100 in PBS). The sections were subsequently incubated with the appropriate primary antibody (dilution 1:500) at 4 °C overnight followed by washing and further incubation with secondary antibodies (dilution 1:1000) for 2 hours in antibody solution (5% goat serum, 0.05% Triton X-100 in PBS). Antibodies included; rabbit anti-interleukin-17 (IL-17) polyclonal antibody (Abcam), rat anti-human leukocyte adhesion glycoprotein Mac-1 α (CD11b) monoclonal antibody (Santa Cruz), and mouse anti-lymphocyte common antigen (CD45) monoclonal antibody (Santa Cruz). Secondary antibodies for visualization included goat anti-rat monoclonal antibodies conjugated with Alexa Fluor 488 and goat anti-rabbit/mouse monoclonal antibodies conjugated with Alexa Fluor 555. Negative controls were obtained by omitting either the primary or secondary antibody and gave no signal (data not shown). Cell nuclei were counterstained with DAPI for the analysis of total cellular infiltration. Slides were examined using a fluorescence microscope (Olympus IX71), and images were captured at a magnification of $\times$ 400 using an Olympus digital camera. The contrast and brightness of images were adjusted using Adobe Photoshop CS5 software (Adobe Systems Inc., San Jose, CA). Subsequently, images were quantified using NIH (National Institute of Health) ImageJ software (Bethesda, MD) [31].

2.2.5. RNA extraction and cDNA synthesis

At the 21st day of EAE induction, animals were euthanized as described in the previous section. Thereafter, mice were transcardially perfused with PBS, the brains were dissected, cerebral

cortex was isolated and snap-frozen in liquid nitrogen. The total RNA was isolated from frozen tissue homogenates using a RNeasy Mini Kit (Qiagen, Germany). The purity and concentration of the extracted RNA were determined by Nanodrop 1000 (Thermo- Scientific, USA). The cDNA was transcribed from one microgram of the total RNA using a PrimeScript™ 1st strand cDNA synthesis kit (Takara, Japan) according to the manufacturer's instruction.

2.2.6. Quantitative real-time PCR.

The mRNA levels of the target genes including; matrix metalloproteinase 2 and 9 (MMP2 and MMP9), tissue inhibitor of matrix metalloproteinase (TIMP-1) and hypoxanthine Phosphoribosyl transferase 1 (HPRT-1), as a normalizer, were measured by quantitative real-time PCR (qRT-PCR) using a step-one system (Applied Biosystems, USA). The sequence of specific primers used for measurement of MMP2, MMP9, TIMP-1, and HPRT1 levels as well as length of amplified fragments were as follows: HPRT1 (5'-CAGCGTCGTGATTAGTGA-3' and 5'-GGTCAGCAAAGAACTTATAGC-3', 215 bp), MMP2 (5'-GACGCTGCCTTTAACTGGAGT-3' and 5'-TCCAGGTTATCAGGGATGGCA-3', 270 bp), MMP9 (5'-CCTGGAAGTCACACAACGTCT-3' and 5'-GCAGGAGGTCATAGGTCACG-3', 135 bp), and TIMP-1 (5'-CTATAGTGCTGGCTGTGGGGT-3' and 5'-CAGGCAAAGTGATCGCTCTGG-3', 365 bp). All PCR amplifications were performed in duplicate reactions utilizing 0.2 µl volume microtubes. The reaction mixture comprised of 5 pmol of specific primers, and 10 µl of Master Mix SYBR Green (2x qPCR Master Mix with SYBR Green I and Rox) in a total volume of 20 µl. The PCR protocol was accomplished as follows: an initial melting step at 95 °C for 10 min to activate DNA Taq polymerase, 40 two-step cycles with 15 s at 95 °C for denaturation followed by annealing and extension at 60 °C for 1 min. The melting curve was tested to ensure the specificity of PCR products. To determine the

relative quantification of gene expression, mean Ct value for each duplicate measurement were used using $2^{-\Delta\Delta C}$ [32].

2.2.7. Estimation of circulating inflammatory biomarkers

At the 21st day of EAE induction, animals were euthanized as described earlier. Thereafter, blood obtained via cardiac puncture and plasma was prepared. Expression levels of tumor necrosis factor-alpha (TNF α) and interleukin 1 β (IL1 β) were measured in the plasma using commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Europe, Abingdon, UK). The cytokine concentrations of respective samples were quantified by standard curves prepared by recombinant cytokines of known concentrations [33]. Samples were quantified only if the absorbance fell on the linear portion of the standard curve. Each sample was run in triplicate.

2.2.8. Statistics

For quantitative measurement, the groups were analyzed using one-way analysis of variance (ANOVA) or the unpaired t-test. A Bonferroni post hoc test for multiple group comparisons was used when appropriate. Each experiment was repeated at least three times and the results were considered significant at $P < 0.05$.

3. Results

3.1. *In silico* results

3.1.1. Sequence 83-109 of IFN β was considered for rational peptide design

Several highly conserved regions were determined in the sequence of IFN β after performing a multiple sequence alignment between human, mouse and rat IFN β . However, the most conserved region is located from amino acids 83 to 109 of the human IFN β sequence (**Fig. 1A**). Sequence alignment of this region in human, mouse and rat revealed 70.37% identity and 85.18% similarity between these species. Surprisingly, a sequence alignment of IFN β and SYR6 deciphered that the peptide SYR6 had a significant similarity to the sequence 95-109 of IFN β , precisely within the candidate site (**Fig. 1B**). As SYR6 has been reported as a peptide mimetic of IFN β with anti-viral activity, the chosen conserved region may presumably play a crucial role in biological activity of IFN β .

Fig. 1. Sequence alignment results. (A) Multiple sequence alignment of human (UniProt ID: P01574), mouse (UniProt ID: P1575) and rat (UniProt ID: P70499) IFN β . The most conserved region is shown in a red box. (B) Sequence alignment of human IFN β and the SYR6 peptide.

Further functional studies utilizing the ConSurf web tool revealed that the selected region comprises several functionally and structurally conserved residues that are expected to have a

central role in IFN β -IFNAR1 interactions (**Fig. 2A**). Structural assignments of IFN β illustrated that the candidate site encompasses a side of the protein that is built up by α -helices 4, 5 and 6, and two loop spacers (**Fig. 2B**). Interestingly, detailed interpretation of murine Ifnar1 in complex with Ifn β using PDBsum and Ligplot+ tools revealed that the residues of the chosen region are apparently involved in the binding of Ifn β to Ifnar1 [34]. The murine Ifn β -Ifnar1 complex, along with regions of Ifn β which are involved in binding to Ifnar1, is depicted in **Fig. 2C**. This finding was also corroborated by a blind docking between human IFN β and IFNAR1 (**Fig. 2D**).

Fig. 2. Detailed interpretation of the candidate binding site of IFN β . (A) Cross-species conservation scores of IFN β obtained from ConSurf. The candidate region used for peptide design is indicated in a black box. The conservation scale used for visualization of the protein is shown on the left. (B) Surface and cartoon representation of 3D structure of human IFN β along with the candidate binding site (red). α 1- α 8: α -helix 1 to α -helix 8. (C) Murine Ifn β -Ifnar1 (left) and docked human IFN β -IFNAR1 (right) complexes obtained from PDB (ID: 3WCY) and docking results, respectively. IFN β is shown in gray and subdomains 1, 2, 3 and 4 of IFNAR1 are indicated in green, blue, yellow and salmon colors, respectively. The binding site and close-up view of residues involved in IFN β -IFNAR1 interactions are shown in red color. SD1-SD4: Fibronectin type III domains of the extracellular domain of IFNAR1, named subdomains 1-4.

Superimposition of alpha-carbon backbones of murine *Ifnar1* and human IFNAR1 (RMSD = 2.63 Å) as well as murine *Ifnβ* and human IFNβ (RMSD = 2.06 Å) demonstrated that there is a notable structural similarity between the proteins of murine and human implying the similarity in their interaction mode (**Fig. 3A, B**). Consequently, the predominant binding sites obtained from published crystallography data (PDB ID: 3WCY) as well as computational docking information were integrated for conducting a high-resolution docking between human IFNβ and IFNAR1. The binding site of IFNβ is located between α-helix 4 and α-helix 6 of the protein, whereas the main *Ifnβ*-binding site of *Ifnar1* is between SD2 and SD3 (**Fig. 3C, D**). Following prediction of 3D structures of the initial peptide (MLQNGRALRKQNFLNTGLRKLGHENLL) and MSPEP27 (MLQNGRDVRKRGFSDTGLRKLGRVNLL) by PEP-FOLD (**Fig. 3E**), a high-resolution docking was performed between each peptide and IFNAR1. The solution structure of SYR6 peptide was downloaded from PDB (PDB ID: 1ID7) and docked with IFNAR1.

Fig. 3. Results of structural alignment, binding sites evaluations and prediction of 3D structures of the peptides. (A) Structural alignment of murine IFN β (light green) and human IFN β (gray), and (B) murine IFNAR1 (colored) and human IFNAR1 (gray). SD1, SD2 and SD3 of IFNAR1 are illustrated with green, blue and yellow colors, respectively. (C) Close-up view of the main binding site of IFN β (orange) and (D) IFNAR1 (yellow). α : α -helix, SD: Subdomain. (E) 3D structure of the initial peptide, MSPEP27 and SYR6 obtained from PEP-FOLD and PDB database.

3.1.2. MSPEP27 is precisely bound to IFN β -binding site of IFNAR1

The *in silico* assessments revealed that MSPEP27 is most likely capable of binding to IFNAR1, with approximately similar binding energy compared to IFN β . The K_d of MSPEP27 is, however, four orders of magnitude higher than that of IFN β . Similar to IFN β , calculation of non-bonded connections implicated important roles of electrostatic energies in the interaction selectivity of the peptides with IFNAR1. The initial and SYR6 peptides appear to have less IFNAR1-binding affinity than MSPEP27 (**Table 1**).

Table 1. Molecular docking results of interaction the peptides and IFN β with IFNAR1

Interaction Free Energy Components in complex with IFNAR1 (kcal mol ⁻¹)				
Energy	Initial peptide	MSPEP27	SYR6	IFN β
ClusPro score	-1801.2	-2447.2	-1658.1	-2810.4
HADDOCK score	-78.0 $\pm$ 8.9	-147.3 $\pm$ 3.1	-76.1 $\pm$ 3.1	-162.0 $\pm$ 5.1
Cluster size	34	28	29	47
$\Delta G_{interaction}$	-9.7	-10.8	-9.2	-15.0
$\Delta E_{electrostatic}$	-175.9 $\pm$ 25.5	-313.8 $\pm$ 29.8	-309.1 $\pm$ 31.3	-625.7 $\pm$ 22.1
ΔE_{vdw}	-59.4 $\pm$ 6.9	-57.9 $\pm$ 2.1	-40.3 $\pm$ 4.5	-86.3 $\pm$ 9.6
$\Delta G_{desolvation}$	-9.6 $\pm$ 9.9	-5.1 $\pm$ 4.4	11.2 $\pm$ 2.2	41.8 $\pm$ 9.3
K_d (M)	5.9e-04	6.5e-08	1.9e-07	9.2e-12
Non Interacting Surface (NIS) per property				
%NIS charged	27.54	26.21	28.39	33.31
%NIS apolar	38.42	29.65	31.78	30.82
K_d : Dissociation constant				

Calculation of surface electrostatic potential demonstrated the main binding sites of IFNAR1 and IFN β have two distinct positively- and negatively-charged interfaces (**Fig. 4A, B**). This feature facilitates the binding of IFN β to its receptor. Considering that the majority of IFNAR1 interacting residues (residues 118-136) make negative surface potential, we considered the high positive surface potential as an important criterion for designing IFN β mimetic peptide.

However, there is a negative surface potential in MSPEP27 to interact with positively-charged residues of binding interface of IFNAR1. Moreover, accessible surface area of MSPEP27 (2967.712 Å²) appeared to be higher than initial peptide (2912.525 Å²) postulating its major binding affinity to IFNAR1 (**Fig. 4C**).

Fig 4. Calculation of surface electrostatic potential around the proteins and peptides. Surface electrostatic potential of (A) IFNAR1, (B) IFNβ and (C) initial peptide (up) and MSPEP27 (down). Gray, blue and red colors infer to hydrophobic regions, positive surface potentials and negative surface potentials, respectively. TC: total charge, SA: surface area (Å²).

All examined peptides could precisely bind to the determined binding site of IFNAR1 in silico, between SD2 and SD3 (**Fig. 5**). The number of interfacial contacts (ICs), particularly polar-apolar and charged-polar contacts, between MSPEP27 and IFNAR1 was substantially higher than the initial peptide and SYR6. Total number of connections in MSPEP27-IFNAR1 (62 contacts) were obviously higher than initial peptide-IFNAR1 (49 contacts) and SYR6-IFNAR1 (42 contacts) complexes implicating that MSPEP27 may probably form a stable interaction compared to other studied peptides. The high number of charged-apolar contacts between all studied peptides and IFNAR1 implicate the pivotal role of these interactions in IFNAR1 signaling.

Fig. 5. Results of molecular docking. Molecular docking of (A) the initial peptide, (B) MSPEP27 and (C) SYR6 with IFNAR1. The main IFN β -binding site of IFNAR1 (containing

residues of both SD2 and SD3) is shown in red. **(D)** The number of Interfacial Contacts (ICs) for each peptide-receptor complex have also provided. ICs: Interfacial contacts, ch: charged, po: polar, ap: apolar.

3.1.3. Physicochemical properties of MSPEP27

Calculation of the physicochemical properties of the initial peptide revealed some undesirable features. Thus, several properties of this peptide were improved using some logical amino acid substitutions to derive MSPEP27. The net charge of MSPEP27 at pH 7 (+5) is close to that of IFN β (+4.3). Moreover, MSPEP27 has a significantly higher theoretical pI compared to IFN β , mostly attributable to an increase in arginine and lysine residues. Importantly, the lower values of the instability index and GRAVY of MSPEP27 (instability index = 20.34, GRAVY = -0.696) compared to IFN β (instability index = 49.23, GRAVY = -0.427) postulate that the peptide has higher stability and hydrophilicity than IFN β . In contrast to IFN β , the MSPEP27 sequence showed an absence of aggregation hot spots implying that the bioactivity of the peptide may not be limited due to aggregation. **Table 2** summarizes measured physicochemical properties of the peptides and IFN β .

Table 2. Physicochemical properties of peptides and IFN β .

Parameters	Initial peptide	MSPEP27	SYR6	IFN β
Formula	C ₁₃₉ H ₂₁₆ N ₃₆ O ₄₄ S ₁	C ₁₃₂ H ₂₃₁ N ₄₇ O ₃₇ S ₁	C ₈₂ H ₁₁₈ N ₂₀ O ₂₅	C ₉₀₈ H ₁₄₀₈ N ₂₄₆ O ₂₅₂ S ₇
No. of amino acids	27	27	15	166
Molecular weight (g/mol)	3127.5	3100.64	1783.9	20027.0
Theoretical Pi	4.14	11.83	4.03	8.93
Net charge at pH 7	-2	+5.0	-2	+4.3
Instability index (II)*	76.50 (unstable)	20.34 (stable)	55.75 (unstable)	49.23 (unstable)
GRAVY[#]	0.030	-0.696	-0.100	-0.427
No. of aggregation hot spots	2	0	0	7
Estimated half-life (hour)	30	30	1.9	30
Cell penetrating score	Non-CPP [†]	Non-CPP	Non-CPP	-
Water solubility	Poor	Good	Good	Good
No. of antigenic regions	1	0	0	6

* A peptide with an II value < 40 is classified as stable.

[#]Grand average of hydropathicity

[†]Non-cell penetrating peptide

3.1.4. The dynamics properties of peptide-IFNAR1 complexes

Conformational changes of the peptide-IFNAR1 complexes as well as IFN β -IFNAR1 were assessed using plotted root mean square deviation (RMSD) of the backbone atoms and radius of gyration (Rg). RMSD and Rg values are widely used for evaluating macromolecules stability and rigidity, respectively [35, 36]. Very small (~ 0.25 nm at the first 15-ns and ~ 0.3 nm in the second 15-ns of MD simulations) and invariable RMSD values of the MSPEP27-IFNAR1 complex imply a high stability and low conformational freedom of the peptide-receptor complex during MD simulations. In contrast, RMSD of SYR6 complexes exhibited some structural fluctuations upon binding to IFNAR1 that can be fully attributed to their structure (**Fig. 6A**). Except for a short period, the Rg of MSPEP27 remained approximately 1.95 nm over the 30 ns MD simulations. The Rg of IFN β increased to ~ 2.3 nm, then decreased to stabilized at ~ 2.25 nm. The Rg values of SYR6-IFNAR1 exhibited a sharp increase from ~ 2 nm to ~ 2.05 nm at 20 ns, and then stabilize around this value (**Fig. 6B**). The initial peptide attained a significant distance from IFNAR1 during MD production phase, and therefore was omitted from analyses.

Fig 6. RMSD and Rg plots. (A) RMSD of the backbone atoms and (B) Rg of the IFN β -IFNAR1 (blue), SYR6-IFNAR1 (orange) and MSPEP27-IFNAR1 (green) complexes as a function of the simulation time.

Intriguingly, using an averaged configuration of MSPEP27-IFNAR1 complex during MD simulations, the content of α -helix in MSPEP27 was shown to increase upon binding to IFNAR1 (77%) compared to unbound MSPEP27 (73%). This conformational change of MSPEP27 results in a stable interaction of MSPEP27 with IFNAR1.

3.1.5. MSPEP27 could strongly bind to IFNAR1

For biomacromolecules, such as proteins, fluorescence measurements could provide useful information including the binding of molecules to proteins at the molecular level. When physicochemical conditions of local surroundings of IFNAR1 are changed slightly, its intrinsic fluorescence would become significantly weakened, where some factors such as biomolecule binding could be responsible for the weakening. To determine the quenching mechanism of the

interaction between IFNAR1 and the MSPEP27, the fluorescence studies were carried out at 298 K (**Fig. 7**). As can be seen in **Fig. 7A**, IFNAR1 fluorescence intensity quenched remarkably as MSPEP27 concentrations increased. This trend indicated that MSPEP27 could interact with IFNAR1, implying the formation of MSPEP27-IFNAR1 inclusion complexes. The reduction in fluorescence intensity resulted from the diminution of the fluorescence quantum yield, which in turn was the result of a lowering of the electronic density after the peptide molecule interacted the receptor. This interaction caused the changes in the microenvironment of aromatic fluorophores of the receptor. This interaction caused the changes in the microenvironment of aromatic fluorophores of the receptor.

Fluorescence quenching data from the interaction of the peptide and receptor were analyzed to obtain various binding parameters. The possible number of binding sites n and binding constant K_a can be determined by using equation 1. A plot of $\log [(F_0-F)/F]$ versus $\log [P]$ (**Fig. 7B**) gives a straight line, and the slope equals to n , and the intercept on Y-axis equals to $\log K_a$. The values of K_a and n at 298 K were $3.67(\pm 0.24) \times 10^7 \text{ M}^{-1}$ and $1.17(\pm 0.09)$, respectively. The n and K_a values indicated that IFNAR1 significantly interacts with the peptide, forming equimolar complex. It is evident from K_a value that the binding of the peptide and receptor is enough strong to make a peptide-receptor inclusion complex.

Fig.7. Results of intrinsic fluorescence measurements. (A) Fluorescence emission spectra of IFNAR1 (3 μ M) in the absence and presence of various concentrations of MSPEP27 (0-30 μ M) in 50 mM phosphate buffer and pH 7.4 at 298 K. (B) Linear plot of $\log F_0 - F/F_0$ versus $\log [IFNAR1]$ for quenching of the receptor fluorescence by MSPEP27 at 298 K. Red arrow indicates the range of MSPEP27 concentrations used in this experiment (from 0 to 30 μ M).

3.2. *In vivo* validations

3.2.1. Therapeutic effects of MSPEP27 treatment on the development of progressive EAE

We surveyed whether MSPEP27 (10, 20 mg kg^{-1} , i.v.) in day 14 has a therapeutic effect in the EAE model. Mice were typically observed for 3 weeks, during which mice remained chronically

paralyzed with onset of paralysis between 12 and 16 days after immunization, and maximum score of 3 to 4 in most mice. Typical EAE onset observed 8 to 11 days after immunization, with peak of disease 12 to 17 days after onset for each mouse. MSPEP27 treatment significantly attenuated severity of clinical EAE compared to the vehicle group ($P < 0.05$ and $P < 0.01$). Our results showed that area under curve (AUC) in MSPEP27-treated groups showed a higher decrease as compared with EAE group (**Fig. 8**). Therefore, mice treated with MSPEP27 showed a significant reduction in the clinical score in comparison with vehicle group, indicating that MSPEP27 has a modulatory effect on EAE progression (**Fig. 8**). As expected, mice treated with IFN β (10 or 20 mg kg $^{-1}$, i.v.) showed a significant reduction in the clinical score in comparison with EAE groups. There was no statistically significant difference between effects of MSPEP27 and IFN β on changes in clinical scores during treatments (**Fig 8A, B**). Accordingly, a significant amelioration in clinical severity was observed for both IFN β - and MSPEP27-injected groups in a time-dependent manner. Moreover, raising the dose of MSPEP27 could further protect mice from EAE clinical symptoms. Indeed, our data indicate that an increased in drug dosage for both IFN β and MSPEP27 improves disease protection with approximately same pattern.

Fig 8. Protective effects of MSPEP27 and IFN β in EAE clinical scores. EAE was induced in C57BL/6 mice by immunization with MOG35-55 and injection of pertussis toxin as described in materials and methods. **(A)** EAE mice were treated with MSPEP27 (10, 20 mg kg⁻¹, ICVI) in the 14th day (start of treatment) from day 0 (start of immunization). Mice that received pertussis toxin with MOG (EAE), EAE mice that received PBS (Vehicle), mice that received pertussis toxin without MOG (Sham), EAE mice that treated with different doses of MSPEP27 (MSPEP27-10 and MSPEP27-20). **(B)** EAE mice were treated with IFN β (IFN β -10, IFN β -20) in the 14th days at day 0 (starting immunization). Mice treated with different doses of IFN β (IFN β -10 and IFN β -20). Data are shown the mean clinical score ($\pm$ SEM) of 15 mice per group from

three experiments. AUC= area under curve, *= P-value < 0.05, **= P-value < 0.01 difference from vehicle group.

3.2.2. Change in expression pattern of MMP2, MMP9, TIMP-1, IL1 β and TNF α after

MSPEP27 treatment

To test the MSPEP27 response over the course of the EAE treatment, protein and gene expression of some related genes such as MMP2, MMP9, TIMP-1, IL1 β and TNF α were monitored and compared with IFN β response at the end of the experiment. Treatment with both higher doses (20 mg kg⁻¹) of MSPEP27 and IFN β showed a significant decrease in MMP2 and MMP9 gene expression in the brain tissue compared to the vehicle group ($P < 0.05$) (**Fig 9A, B**). In addition, treatment groups with higher doses showed a significant increase in the TIMP-1 gene expression compared to vehicle group ($P < 0.05$) (**Fig 9C**). After treatment with different concentrations of IFN β and MSPEP27, EAE mice showed a significant decrease in plasma levels of TNF α and IL1 β compared to vehicle group ($P < 0.05$) (**Fig 9D, E**). However, the reduction of these inflammation mediators was more pronounced in IFN β -treated mice.

Fig 9. Real-time qPCR and ELISA results. Effect of different concentrations of IFN β and MSPEP27 treatment on (A) MMP2, (B) MMP9 and (C) TIMP-1 expression as measured by RT-qPCR in brain of EAE mice as well as on concentration of (D) IL-1 β and (E) TNF- α in plasma, as measured by ELISA, in EAE mice. The data are presented as the mean $\pm$ SEM (n=5). *: $p < 0.05$ vs vehicle group, #: $p < 0.05$ vs IFN β group. EAE mice that received PBS (EAE), EAE mice that treated with 20 mg kg $^{-1}$ of MSPEP27 (MSPEP27) or IFN β (IFN β).

3.2.3. Significant reduction of IL-17, CD11b, and CD45 in the cerebral cortex after MSPEP27 treatment

To assessment of inflammatory infiltrates and monitoring brain inflammation, IHC staining for IL-17 as the most important pro-inflammatory cytokine produced by T-helper cells, CD11b as an activated macrophages and microglia marker, and CD45 as a general leukocytes marker were performed. According to the results, IL-17, CD11b, and CD45 positive cells were considerably decreased in mice treated with MSPEP27, particularly in the higher dosage (20 mg kg $^{-1}$).

Quantitative analysis revealed that this reduction occurs in a dose-dependent manner. The percentage of IL-17, CD11b and CD45 positive cells in the mice treated with 20 mg kg⁻¹ of MSPEP27, were all less than half of the untreated EAE mice. In this appraisal, the most significant decrease was observed for CD45, especially when 20 mg kg⁻¹ of MSPEP27 was applied (**Fig 10A**). On the other hand, IL-17, CD11b, and CD45 positive cells were considerably decreased in mice treated with IFN β , particularly in the 20 mg kg⁻¹ dosage (**Fig 10B**). There was no statistically significant difference between effects of MSPEP27 and IFN β on expression of IL-17, CD11b, and CD45 during treatments (**Fig 10A and B**).

Fig 10. Injection of MSPEP27 and IFN β on day 14 post-immunization and evaluation of brain inflammatory responses after 21 days of EAE induction. Upper panel represents the IHC results for IL17 (red), CD11b (green) and CD45 (red). Lower panel **A**, percentage of IL17, CD11b and CD45 positive cells in each μm^2 after MSPEP27 injection. Lower panel **B**, percentage of IL17, CD11b and CD45 positive cells in each μm^2 after IFN β injection. Data are expressed as means $\pm$ SEM (n=5). * comparison to sham, # used for comparison to EAE + vehicle mice (* or # = P-value < 0.05, ** or ## = P-value < 0.01 and *** or ### = P-value < 0.001). Scale bar, 50 μm .

4. Discussions and Conclusions

4.1. Discussions

IFN β -based drugs have revolutionized the treatment of MS by demonstrating a clinically significant decrease in disease progression, reduction of frequency of MS attacks and the number of new lesions that can be detected by magnetic resonance imaging (MRI). These effects presumably occur through the activation of signaling pathways downstream of IFNAR. However, consequences of neutralizing antibody (NAbs) formation against IFN β -based therapies for MS has been a topic of debate for several years [37]. In this study, a novel peptide, called MSPEP27, was designed and developed as a mimetic of human IFN β , for the treatment of MS. It is plausible that this peptide can efficiently modulate the detrimental inflammation mediating the demyelination process. Based on the results of MD simulations, formation of a stable MSPEP27-IFNAR1 complex implies the high potential of MSPEP27 to compete with IFN β for binding to IFNAR1. Consequently, this peptide may also be able to induce a conformational change in IFNAR1 structure required for elicitation of downstream response. Thus, the immunomodulatory effect of MSPEP27 in the EAE model can be attributed to its excellent binding affinity to IFNAR1. Due to its smaller size and better physicochemical properties, however, it can be postulated that the side effects of MSPEP27 therapy will be much lower than that of IFN β . It should be emphasized that further experiments are necessary to determine the favorable dose and administration route.

Owing to their unique physicochemical properties, peptides have found increasing applications in important branches of biological and medical researches. These small compounds are highly selective, efficacious, safe and stable [38]. Therefore, peptides/peptidomimetics are now widely

used in immunomodulation of several autoimmune diseases such as MS, rheumatoid arthritis, systemic lupus as well as HIV infections and cancers [39]. Herein, this approach was used for the treatment of MS to avoid side effects and the intrinsic weaknesses of all IFN β formulations. Like IFN β , MSPEP27 was capable of reducing the expression of crucial inflammatory factors. We suppose that this anti-inflammatory effect of MSPEP27 is mediated through stable interaction of the peptide with IFNAR1. This claim was confirmed using MD simulation that is reliably used for understanding the physical basis of structure-function of macromolecules under physiological conditions [40]. Owing to its smaller size and lack of epitopes, it is reasonable that the activity of MSPEP27 is not limited via production of neutralizing antibodies. This is because of the fact that antibodies usually bind to the native protein with higher affinity than the peptides. However, some controversies have been reported for a number of peptides with stable structures such as cyclized peptides [41]. The linear MSPEP27 does not seem to have a stable structure, similar to IFN β , to elicit neutralizing antibodies. This was further proved by dynamic changes of MSPEP27 conformation during MD simulation. The lower values of the instability index and GRAVY of MSPEP27 implicate the higher stability and hydrophilicity level of the peptide which may translate to longer bioactivity when compared to IFN β . The instability index is measured according to the occurrence of certain dipeptides in a protein sequence. It is now well documented that the occurrence frequency of certain dipeptides is significantly different in the unstable proteins compared to those in the stable ones. Accordingly, instability index values below 40 imply an unstable structure of the assessed protein or peptide [42]. The instability index of MSPEP27 and IFN β was calculated as 24.17 and 49.23, respectively. Positive GRAVY values infer that the protein or peptide is hydrophobic, while a negative GRAVY value shows the hydrophilicity of a protein [43]. Both MSPEP27 and IFN β demonstrated a negative GRAVY

value implying their desire activity in hydrophilic conditions. Unraveling the aggregation hotspots and epitopes on protein surfaces has become an increasingly important factor in designing protein-based drugs [44]. These regions can potentially increase the immunogenicity and reduce the half-life and activity of protein-based drugs. Prediction of aggregation hot spots and epitopes illustrated that IFN β -based design of MSPEP27 had reduced the aggregation hot spots (7 numbers) and antigenic regions (6 numbers) of the peptide to zero.

MMPs are a class of zinc-dependent endopeptidases involved in an array of physiological processes including remodeling of extracellular matrix (ECM), angiogenesis and cytokine regulation. The enzymatic activity of these proteins is precisely regulated by TIMPs, a group of inhibitor proteins consisting of four family members. Studies have shown that up-regulation of MMPs is substantially associated with an increased number of gadolinium-enhancing MRI lesions [45]. In particular, expression of MMP9 has been demonstrated to be elevated around plaques and reaches highest levels during the appearance of clinical symptoms. Furthermore, enhanced MMP2 expression results in chronic progression of MS [46, 47]. In contrast, serum levels of TIMP-1 are significantly lower in RRMS patients than normal matched controls [48]. Recent studies have found that IFN β , a commonly used immunomodulatory agent in MS, can efficiently reduce production of MMP9 by T cells, and thereby decrease transmigration of T lymphocytes across basement membrane barrier [49]. However, TIMP-1 levels have shown to be gradually increased during IFN β -based therapy [50]. The modulatory effect of IFN β on the gelatinolytic activities of MMP2 and MMP9 has also reported by Kurzepa and Stryjecka-Zimmer [51]. Furthermore, IL1 β and TNF α are crucial mediators of inflammatory reactions in the CNS of MS patients [52, 53]. On the other hand, biopsies from MS patients taken for diagnostic reasons showed that cortical demyelination is already present at early stages of the disease and,

when this happens, the lesions show profound lymphocytic infiltration similar to that seen in active white matter lesions [54]. EAE is composed of an induction phase, which involves the loading of myelin specific T cells following immunization with myelin individual proteins, and an effector phase, which involves the migration of activated myelin-specific T cells to the CNS and triggering of neuroinflammation [28]. Since, in MOG-induced EAE both spinal cord and forebrain regions are attacked simultaneously [55], in this experiment we evaluated cerebral cortex region in C57BL/6 mice by focusing on inflammatory responses, and trans-endothelium migration of immune cells.

In agreement with previous findings, analysis of cerebral cortex in EAE mice revealed that treatment with both MSPEP27 and IFN β significantly decreased mRNA expression of MMP2 and MMP9 and protein concentration of IL1 β and TNF α in compare with EAE group.

Interpretation of IHC on the cerebral cortex of EAE mice revealed that the groups treated with MSPEP27 were remarkably different from the untreated vehicle group in a number of cells presenting IL17, CD11b, and CD45. A remarkable and significant decrease was observed in the expression of all three aforementioned proteins after treatment with both MSPEP27 and IFN β . Furthermore, we noticed that this reduction was dose-dependent and that 20 mg kg⁻¹ of the peptide could attenuate the expression of these markers more efficiently than 10 mg kg⁻¹. IL17 is a cytokine that is produced by activated T cells which are differentiated to Th17 cells and have been implicated in the pathogenesis of neuroinflammatory diseases [56]. Durelli and colleagues have been reported that the percentage of Th17 cells in patient with active MS was sevenfold more than healthy subjects and IFN β treatment could reduce the number of these cells [57]. Likewise, MSPEP27 treatment successfully reduced the number of IL17 expressing cells in our experiment. Moreover, it is also established that the expression of CD11b on

monocytes/macrophages and other pathogenic cells is essential for EAE creating [58]. Our results indicated a significant decrease in the number of CD11b presenting cells (infiltrating macrophage or resident microglia) in the MSPEP27-treated groups compared to the EAE group. Since CD45 is a marker of inflammatory cells, including leukocytes, macrophages and T lymphocytes, the effect of MSPEP27 on expression of this cell marker was also evaluated. Surprisingly, MSPEP27 treatment could significantly reduce CD45 expression in comparison with EAE group.

It has been accepted that IFN β exerts its therapeutic function in part through anti-proliferative, anti-viral, and particularly, immunomodulatory actions [59]. Moreover, intrastriatal injection of IFN β has been shown to preserve the BBB integrity, block the infiltration of inflammatory cells, and decrease infarct size in a middle cerebral artery occlusion model [60]. Furthermore, in a model of transient focal stroke, it was reported that intravenous tail injections of IFN β failed to provide protection. It appears that IFN β is unable to cross BBB; thus, methods to circumvent the BBB are needed in order for IFN β to be effective [61]. Although, IFN β is unable to cross BBB, permeability of BBB to MSPEP27 and possibility of MSPEP27 action within the CNS after systemic administration should be evaluated for either an "*in vitro*" study using an artificial model of the BBB or the "*in vivo*" studies in an animal model of MS in the near future.

To address whether MSPEP27 can effectively reduce the undesirable side effects of IFN β in the body, the subsequent experiments should be performed in future investigations. Assessing the biological half-life of the MSPEP27 versus IFN β in mice, assessing any potential of MSPEP27 versus IFN β to generate neutralizing antibodies, determining if leukopenia was less severe after treatment with MSPEP27 versus IFN β , and determining the long-term potency of the MSPEP27

versus IFN β beyond 21 days could be supportive of our recent findings. Also the idea being greater potency if the MSPEP27 could decrease the number of injections required to maintain the suppressive phenotype in MS. One of the disadvantages of IFN β therapies is the need for parenteral therapy. Thus it would be supportive that know whether MSPEP27 can work orally, or, at least, by a less invasive delivery route such as subcutaneous or intraperitoneal delivery.

4.2. Conclusions

In conclusion, to the best of our knowledge, this is the first study reporting the rational design of an IFN β mimetic peptide, called MSPEP27, for application in MS therapy. When compared to IFN β , MSPEP27 showed relatively similar biological effects on the suppression of inflammatory factors. The oral bioavailability and BBB permeability of this peptide need to be investigated further. Moreover, validation of *in silico* predictions (e.g., physicochemical properties of MSPEP27) by experimental assays would definitely increase the reliability of this study. Unfortunately, due to some limitations, we could not perform further experiments in this study. However, the results of this study suggest that our peptide-based approach can be considered as an alternative strategy for treatment of MS and most likely to overcome some of the adverse effects associated with IFN β -based therapies.

5. Authors' Contribution

MP: Study design and concept, main manuscript writing and bioinformatics analyses. MA: Manuscript writing, bioinformatics analyses. NS, HA, MM: *In vivo* analyses. AT, PG: The peptide-receptor binding assay, MF A, SS, SS A, M NA: Bioinformatics analyses and manuscript revision. DK, MH N, AM: English language editing and scientific revision of the manuscript.

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9. Conflict of interest

No conflict of interest to be declared by any of the authors.

10. References

1. Pinheiro, M.A.L., et al., *Immune cell trafficking across the barriers of the central nervous system in multiple sclerosis and stroke*. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2016. **1862**(3): p. 461-471.
2. Wingerchuk, D.M. and J.L. Carter. *Multiple sclerosis: current and emerging disease-modifying therapies and treatment strategies*. in *Mayo Clinic Proceedings*. 2014. Elsevier.
3. Alexander, S.P., *Therapeutic potential of cannabis-related drugs*. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2016. **64**: p. 157-166.
4. Limmroth, V., N. Putzki, and N.J. Kachuck, *The interferon beta therapies for treatment of relapsing-remitting multiple sclerosis: are they equally efficacious? A comparative review of open-label studies evaluating the efficacy, safety, or dosing of different interferon beta formulations alone or in combination*. Therapeutic advances in neurological disorders, 2011: p. 1756285611413825.
5. Walther, E. and R. Hohlfeld, *Multiple sclerosis side effects of interferon beta therapy and their management*. Neurology, 1999. **53**(8): p. 1622-1622.
6. van Beers, M.M., et al., *Aggregated recombinant human interferon Beta induces antibodies but no memory in immune-tolerant transgenic mice*. Pharm Res, 2010. **27**(9): p. 1812-24.
7. Reuss, R., *PEGylated interferon beta-1a in the treatment of multiple sclerosis—an update*. Biologics, 2013. **7**: p. 131-138.

8. Poorebrahim, M., et al., *Rational design of DKK3 structure-based small peptides as antagonists of Wnt signaling pathway and in silico evaluation of their efficiency*. PloS one, 2017. **12**(2): p. e0172217.
9. Iman, M., et al., *HOXB7 and Hsa-miR-222 as the Potential Therapeutic Candidates for Metastatic Colorectal Cancer*. Recent Pat Anticancer Drug Discov, 2016. **11**(4): p. 434-443.
10. Sato, A. and S. Sone, *A peptide mimetic of human interferon (IFN)-beta*. Biochem J, 2003. **371**(Pt 2): p. 603-8.
11. de Weerd, N.A., et al., *Structural basis of a unique interferon-[beta] signaling axis mediated via the receptor IFNAR1*. Nature immunology, 2013. **14**(9): p. 901-907.
12. Larkin, M.A., et al., *Clustal W and Clustal X version 2.0*. Bioinformatics, 2007. **23**(21): p. 2947-2948.
13. Waterhouse, A.M., et al., *Jalview Version 2—a multiple sequence alignment editor and analysis workbench*. Bioinformatics, 2009. **25**(9): p. 1189-1191.
14. Glaser, F., et al., *ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information*. Bioinformatics, 2003. **19**(1): p. 163-164.
15. Maiti, R., et al., *SuperPose: a simple server for sophisticated structural superposition*. Nucleic acids research, 2004. **32**(suppl 2): p. W590-W594.
16. Zhang, Y. and J. Skolnick, *TM-align: a protein structure alignment algorithm based on the TM-score*. Nucleic acids research, 2005. **33**(7): p. 2302-2309.
17. DeLano, W.L., *The PyMOL molecular graphics system*. 2002.
18. Comeau, S.R., et al., *ClusPro: an automated docking and discrimination method for the prediction of protein complexes*. Bioinformatics, 2004. **20**(1): p. 45-50.
19. De Vries, S.J., M. Van Dijk, and A.M. Bonvin, *The HADDOCK web server for data-driven biomolecular docking*. Nature protocols, 2010. **5**(5): p. 883.
20. Chill, J.H., et al., *The human type I interferon receptor: NMR structure reveals the molecular basis of ligand binding*. Structure, 2003. **11**(7): p. 791-802.
21. Laskowski, R.A. and M.B. Swindells, *LigPlot+: multiple ligand–protein interaction diagrams for drug discovery*. Journal of chemical information and modeling, 2011. **51**(10): p. 2778-2786.
22. Maupetit, J., P. Derreumaux, and P. Tuffery, *PEP-FOLD: an online resource for de novo peptide structure prediction*. Nucleic acids research, 2009: p. gkp323.
23. Lauck, F., et al., *RosettaBackrub—a web server for flexible backbone protein structure modeling and design*. Nucleic acids research, 2010. **38**(suppl 2): p. W569-W575.
24. Pronk, S., et al., *GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit*. Bioinformatics, 2013: p. btt055.
25. Vangone, A. and A.M. Bonvin, *Contacts-based prediction of binding affinity in protein-protein complexes*. 2015. **4**: p. e07454.
26. Mehraban, M.H., et al., *Binding study of novel anti-diabetic pyrimidine fused heterocycles to β -lactoglobulin as a carrier protein*. Colloids and Surfaces B: Biointerfaces, 2013. **112**: p. 374-379.
27. Pettersen, E.F., et al., *UCSF Chimera—a visualization system for exploratory research and analysis*. Journal of computational chemistry, 2004. **25**(13): p. 1605-1612.
28. Ramroodi, N., et al., *Prophylactic effect of BIO-1211 small-molecule antagonist of VLA-4 in the EAE mouse model of multiple sclerosis*. Immunological investigations, 2015. **44**(7): p. 694-712.
29. Tullman, M.J., *A review of current and emerging therapeutic strategies in multiple sclerosis*. The American journal of managed care, 2013. **19**(2 Suppl): p. S21-7.
30. Peiris, M., et al., *A model of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice for the characterisation of intervention therapies*. Journal of neuroscience methods, 2007. **163**(2): p. 245-254.

31. Solati Sarvandi, S., et al., *In vitro differentiation of rat mesenchymal stem cells to hepatocyte lineage*. Iranian journal of basic medical sciences, 2015. **18**(1): p. 89-97.
32. Mard, S.A., et al., *Antisecretory effect of hydrogen sulfide on gastric acid secretion and the involvement of nitric oxide*. BioMed research international, 2014. **2014**.
33. Sanadgol, N., et al., *Effect of palmitic acid and linoleic acid on expression of ICAM-1 and VCAM-1 in human bone marrow endothelial cells (HBMECs)*. Archives of medical science: AMS, 2012. **8**(2): p. 192.
34. Laskowski, R.A., *PDBsum: summaries and analyses of PDB structures*. Nucleic acids research, 2001. **29**(1): p. 221-222.
35. Simmerling, C., B. Strockbine, and A.E. Roitberg, *All-atom structure prediction and folding simulations of a stable protein*. Journal of the American Chemical Society, 2002. **124**(38): p. 11258-11259.
36. Lobanov, M.Y., N. Bogatyreva, and O. Galzitskaya, *Radius of gyration as an indicator of protein structure compactness*. Molecular Biology, 2008. **42**(4): p. 623-628.
37. Cohen, B.A., et al., *The implications of immunogenicity for protein-based multiple sclerosis therapies*. Journal of the neurological sciences, 2008. **275**(1): p. 7-17.
38. Fosgerau, K. and T. Hoffmann, *Peptide therapeutics: current status and future directions*. Drug discovery today, 2015. **20**(1): p. 122-128.
39. Gokhale, A.S. and S. Satyanarayanajois, *Peptides and peptidomimetics as immunomodulators*. 2014.
40. Karplus, M. and J.A. McCammon, *Molecular dynamics simulations of biomolecules*. Nature Structural & Molecular Biology, 2002. **9**(9): p. 646-652.
41. Camacho, C.J., Y. Katsumata, and D.P. Ascherman, *Structural and thermodynamic approach to peptide immunogenicity*. PLoS Comput Biol, 2008. **4**(11): p. e1000231.
42. Guruprasad, K., B.B. Reddy, and M.W. Pandit, *Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence*. Protein engineering, 1990. **4**(2): p. 155-161.
43. Kyte, J. and R.F. Doolittle, *A simple method for displaying the hydropathic character of a protein*. J Mol Biol, 1982. **157**(1): p. 105-32.
44. Pelay-Gimeno, M., et al., *Structure-Based Design of Inhibitors of Protein–Protein Interactions: Mimicking Peptide Binding Epitopes*. Angewandte Chemie International Edition, 2015. **54**(31): p. 8896-8927.
45. Vincenti, M.P., *The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes*. Matrix Metalloproteinase Protocols, 2001: p. 121-148.
46. Avolio, C., et al., *Serum MMP-2 and MMP-9 are elevated in different multiple sclerosis subtypes*. Journal of neuroimmunology, 2003. **136**(1): p. 46-53.
47. Kieseier, B.C., et al., *Matrix metalloproteinase-9 and -7 are regulated in experimental autoimmune encephalomyelitis*. Brain, 1998. **121**(1): p. 159-166.
48. Boz, C., et al., *Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase (TIMP-1) in patients with relapsing–remitting multiple sclerosis treated with interferon beta*. Clinical neurology and neurosurgery, 2006. **108**(2): p. 124-128.
49. Yushchenko, M., et al., *Interferon- β -1 b decreased matrix metalloproteinase-9 serum levels in primary progressive multiple sclerosis*. Journal of neurology, 2003. **250**(10): p. 1224-1228.
50. Karabudak, R., et al., *Effect of interferon β -1a on serum matrix metalloproteinase—9 (MMP-9) and tissue inhibitor of matrix metalloproteinase (TIMP-1) in relapsing remitting multiple sclerosis patients*. Journal of neurology, 2004. **251**(3): p. 279-283.
51. Kurzepa, J. and M. Strycka-Zimmer, *The effect of interferon beta-1a on MMP-2 and MMP-9 proteolytic activity*. Folia Biol (Praha), 2007. **53**(6): p. 220-1.

52. Heidary, M., et al., *The analysis of correlation between IL-1B gene expression and genotyping in multiple sclerosis patients*. J Neurol Sci, 2014. **343**(1-2): p. 41-5.
53. Lim, S.-Y. and C.S. Constantinescu, *TNF- α : A paradigm of paradox and complexity in multiple sclerosis and its animal models*. Open Autoimmun J, 2010. **2**: p. 160-170.
54. Lucchinetti, C.F., et al., *Inflammatory cortical demyelination in early multiple sclerosis*. New England Journal of Medicine, 2011. **365**(23): p. 2188-2197.
55. Girolamo, F., et al., *Cerebral cortex demyelination and oligodendrocyte precursor response to experimental autoimmune encephalomyelitis*. Neurobiology of disease, 2011. **43**(3): p. 678-689.
56. Constantinescu, C.S., et al., *Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS)*. Br J Pharmacol, 2011. **164**(4): p. 1079-106.
57. Durelli, L., et al., *T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta*. Ann Neurol, 2009. **65**(5): p. 499-509.
58. Bullard, D.C., et al., *Critical Requirement of CD11b (Mac-1) on T Cells and Accessory Cells for Development of Experimental Autoimmune Encephalomyelitis*. The Journal of Immunology, 2005. **175**(10): p. 6327-6333.
59. Kasper, L.H. and A.T. Reder, *Immunomodulatory activity of interferon-beta*. Annals of clinical and translational neurology, 2014. **1**(8): p. 622-631.
60. Veldhuis, W.B., et al., *Interferon-beta prevents cytokine-induced neutrophil infiltration and attenuates blood-brain barrier disruption*. Journal of Cerebral Blood Flow & Metabolism, 2003. **23**(9): p. 1060-1069.
61. Maier, C.M., et al., *Interferon- β fails to protect in a model of transient focal stroke*. Stroke, 2006. **37**(4): p. 1116-1119.

Highlights

- In this study a peptide mimetic of IFN β , called MSPEP27, was computationally designed and improved for treatment of multiple sclerosis (MS).
- MSPEP27 has favorable physicochemical properties, high stability and high binding affinity to IFNAR1.
- Similar to IFN β , the MSPEP27 could significantly attenuate production of proinflammatory factors in vivo.