



Discovery of Novel Peptidomimetics for Brain-Derived Neurotrophic Factor using Phage Display Technology

Fatemeh Nafian^a, Mohammad Javad Rasaee^{a*}, Shahin Yazdani^{b,c}, Zahra Soheila Soheili^d

^aDepartment of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

^bOcular Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

^cOphthalmic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ⁴Department of Molecular Medicine, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Abstract

Brain-Derived Neurotrophic Factor (BDNF) is a neuroprotectant candidate for neurodegenerative diseases. However, there are several clinical concerns about its therapeutic applications. In the current study, we selected BDNF-mimicking small peptides from phage-displayed peptide library as alternative molecules to the clinical challenges. The peptide library was screened against BDNF receptor (Neurotrophic Tyrosine Kinase Receptor Type 2, NTRK2) and evaluated by ELISA. Polyclonal phage ELISA indicated that target populations were enriched round by round and the panning process was truly effective. The results of monoclonal phage-ELISA showed that all clones had principally bound to NTRK2 but fifteen best clones were sequenced, which twelve of them have SGVYKVA YD WQH (peptide 1) sequence, two pairs were GLH TSATNLYLH (peptide 2), and TVLSHPSTATLI (peptide 3) and one sequence was QQRPYVQDLRLI (peptide 4). Alignment of these peptides and BDNF sequence showed that the resulting peptides conformationally mimicked loop 2 (E40-KVPVSKGQLK-Q51) of BDNF. This region of BDNF is responsible for specific receptor binding and biological activity. According to the similarity of these peptides with BDNF, they could be considered as novel peptidomimetics with therapeutic properties. In addition, modeled peptides were submitted to Protein Model Data Base (peptides 1, 2, 3 and 4 as PMDB ID: PM0081104, PM0081105, PM0081106, PM0081107, respectively).

Keywords, Brain-Derived Neurotrophic Factor; Neurotrophic Tyrosine Kinase Receptor Type 2; phage display peptide library; peptidomimetic; agonist; neuroprotectant.

1. Introduction

Brain-Derived Neurotrophic Factor (BDNF) exerts powerful effects on downstream cell signaling involved in survival

and neuron function in the CNS through the NTRK2 receptor. BDNF directly involves in dendritic morphology and branching, as well as synaptic plasticity and long term potentiation (1). In this manner, BDNF

Corresponding Author: Mohammad Javad Rasaeae, Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Tel: +98-2182884513

E-Mail: rasaeae_m@modares.ac.ir

Cite this article as: Nafian F, Rasaeae M, Yazdani S, Soheili Z, Discovery of Novel Peptidomimetics for Brain-Derived Neurotrophic Factor using Phage Display Technology, 2018, 14 (4): 9-20.

influences potential of memory and learning, as Nagahara and colleagues noted (2). A reduced expression of mRNA and BDNF protein in some neurodegenerative diseases such as Parkinsonian and Alzheimer (3-5) is demonstrated. Following tremendous therapeutic potential of BDNF, it is suggested as an interesting therapeutic candidate in neurological and psychiatric diseases (2, 6-16). However, BDNF is not well suited for use as a drug due to inappropriate pharmacokinetic properties such as low oral bioavailability, poor penetration through cellular membranes, and short plasma half-life.

An alternative approach to circumvent therapeutic problems of BDNF is the use of small synthetic mimotopes that selectively activate the NTRK2 receptor. On the other hand, these small molecules should ideally enhance cell survival by targeting survival receptor (NTRK2), in combination with other tyrosine kinase receptors, while having minimal lethal interactions with death receptor (p75NTR). So far, numerous efforts have been devoted to the *in vitro* design of small mimetic molecules specific for NTRK2. Fragment-based mimicry strategies have been extensively explored to generate NTRK2

agonists (17-19). However, none of these peptidomimetics could mimic BDNF function completely (20).

In this study, several peptides with high affinity to the NTRK2 (but not p75NTR) receptor were identified by phage display technology as BDNF-mimicking small peptides. Phage library displays millions of random peptides on the surface of phage particles which can be selected by specific ligands, and identified by DNA sequencing. It seems that phage display technology could provide an appropriate approach for us to select looked-for peptides and investigate them as peptidomimetics of BDNF protein.

2. Materials and Methods

2.1. Isolation of Phage Displayed Peptides against Recombinant NTRK2

The Ph.D-12 (*New England Biolabs*, Herts, UK) peptide library (2×10^{11} pfu) was added to 600 ng of recombinant human NTRK2 (Fc-His NTRK2) (*Creative Biomart*, New York, NY, USA) in 200 μ L of TBS/T (0.1% (v/v) Tween 20) and incubated for 2h. Then, 50 μ L of protein G magnetic beads (*Protein G Mag Sepharose*, *GE Healthcare Ltd*, UK) were added. After 1 h of incubation, the tube was placed on an external magnet and the supernatant was removed. The magnetic beads were washed 8 times with 1 mL of TBS/T (0.1%) and subsequently the bound phage particles were eluted with 1 mL of 0.1 M glycine-HCl (pH 2.2) containing 1 mg mL⁻¹ BSA (10 min incubation). In order to neutralize the eluted phage particles, the supernatant was added to a tube containing

150 μL of 1 M Tris-HCl (pH 9.1) and amplified in *E. coli* ER2738 host as instructed by the manufacturer.

One part (approximately 2×10^{11} pfu) of the amplified phage stock from the first round was pre-incubated with the beads in the absence of target to exclude protein G-binding phage particles. Then, the unbound phage fraction was incubated with 600 ng of recombinant human NTRK2 as mentioned above. Once again, only NTRK2 bound phage particles were fished by incubating the complex with magnetic beads. The stringency of panning was increased after the second round of panning using reduced concentration of target protein and increased detergent concentration. Also, a further round of bio-panning was carried out in the same manner as the first round, except that the amplified phage particles were pre-incubated with recombinant human p75NTR (NGF R/TNFRSF16 Fc Chimera, R&D System, Minneapolis, MN, USA) to improve and optimize subtractive bio-panning method.

2.2. Specificity of Ph.D-12 Peptide Phage against Recombinant NTRK2

The specificity of polyclonal phage particles was measured after each round of panning by enzyme-linked immunosorbent assay (ELISA). Briefly, wells of microtiter plate were coated with $10 \mu\text{g mL}^{-1}$ of NTRK2, p75NTR (as non-specific bindings) or BSA (as a negative control). Afterwards, approximately 5×10^{10} phage particles were added per well. After incubation for 1 h at 37°C , the plate was washed 3 times and $50 \mu\text{l}$ of an anti-M13

antibody-HRP conjugate (GE Healthcare) in EIA buffer was added and incubated. Finally, $50 \mu\text{l}$ of a substrate chromogen (TMB) was added and the plate was incubated at room temperature in the dark. Absorbance at 450 nm was measured using MultiskanTM ELISA reader.

The specificity of individual phage plaques was also measured by affinity capture of the NTRK2-phage complex onto protein A-coated plate. A 100-fold representation of the library (e.g., 2.5×10^{11} pfu for a library) was complexed with recombinant NTRK2, Fc-His (by a 30-nM final concentration) in $100 \mu\text{L}$ TBS/T (0.1%). The pre-formed target-phage complex was transferred to the protein A-coated plate. The plate was incubated for 2 h under 37°C , washed 3 times with TBS/T (0.05%, pH 7.2) followed by the ELISA protocol.

2.3. Identification of NTRK2-Specific Phage Displayed Peptide Clone

Single-strand DNA was extracted from individual phage plaques after final round. PCR was performed to verify the presence of inserts in the phage DNA using the primers 5'TTAGTCCTCAAAGCCTCTG3'(Forward) and 5'CAAGCCCAATAGGAACCC3'(Reverse), which yielded products of the desired 550bp size for all the clones. The inserted regions of 15 clones were sequenced using these primers. Multiple alignments of the resulting amino acid sequences with a segment of the human BDNF sequence were performed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo>) and

confirmed obvious similarities by MEGA6 (21).

We used PEP-FOLD 3.1 server to *de novo* model 3D structures of sequenced peptides. PEPFOLD uses HMMSA profiles and assembles the predicted fragments via the coarse-grained force field (<http://mobylipe.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3>). This server estimates conformations for linear peptides from 5 to 50 amino acids (22, 23). The models were analyzed and sorted according to score values. These models were submitted to Protein Model Data Base (PMDB: <https://bioinformatics.cineca.it/PMDB>).

3. Results and Discussion

In the current study, a strategy was adopted using lower amounts of target protein and more stringent washing conditions in sequential screening rounds. This strategy resulted in successful panning with higher binding affinities compared to the natural ligand (BDNF). It was also aimed to design a BDNF peptidomimetic by selective binding to the NTRK2 protein as a survival receptor while avoiding interaction with the p75NTR protein as a death receptor. Accordingly, the undesired phage particles, that were specifically bound to the p75NTR protein by employing negative selection were removed. After four rounds of panning, the output/input ratio of phages recovered for each round was used to determine the phage recovery efficiency. As estimated, the yield of NTRK2-specific phages in the first and second rounds

of panning was very low but amplification and reselection of these particles in the next rounds resulted in ly increased recovery.

After each round of selection, polyclonal phage-ELISA was performed by incubating the eluted phage population with NTRK2 as target protein, along with p75NTR (for non-specific bindings control), and BSA (as a non-related control). ELISA signals for each antigen (x axis) suggested that target populations were enriched round by round. On the other hand, the eluted phage of the last rounds bind to NTRK2 with greater affinity towards the first rounds and the panning process was truly effective (Figure 1). As a result, the specific signal of the last round was significantly increased; however the unenriched library produced weak signals.

Individual phage plaques were randomly selected from the plates used for titration of the last round recoveries. The binding affinity was measured as *ELISA* binding unit [*optical density (OD)* at 450/630 nm] net values after subtraction of *OD* values of controls. The results of monoclonal phage-ELISA indicated that all clones had principally bound to NTRK2 (binding affinity above 2.1), as shown in figure 2. Clones 1, 3, 10, 14, 16, 17, 19, 20, 22, 23, 24, 28, 30, 31, and 32 had a higher binding capacity to the antigen (quadruple asterisks with a significant difference in figure 2) and were selected for DNA amplification and sequencing of inserted peptide regions (Figure 3). In fact, the results of monoclonal phage-ELISA determined that most phage displayed peptides (approximately 86.1%) were NTRK2-specific; however various clones

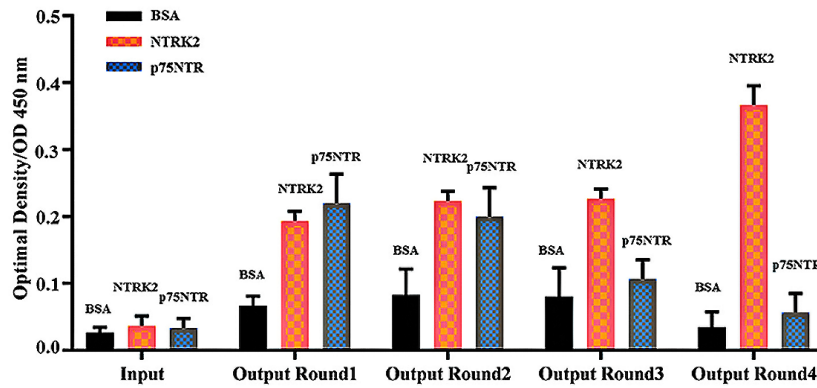


Figure 1. Polyclonal phage ELISA and evolution of population specificity toward NTRK2. Unlike negative controls, absorbance values against the target protein increased after each panning round. Immobilized p75NTR protein and BSA were used as negative controls. Absorbance values (450 nm) are shown as mean \pm SD for three independent measurements.

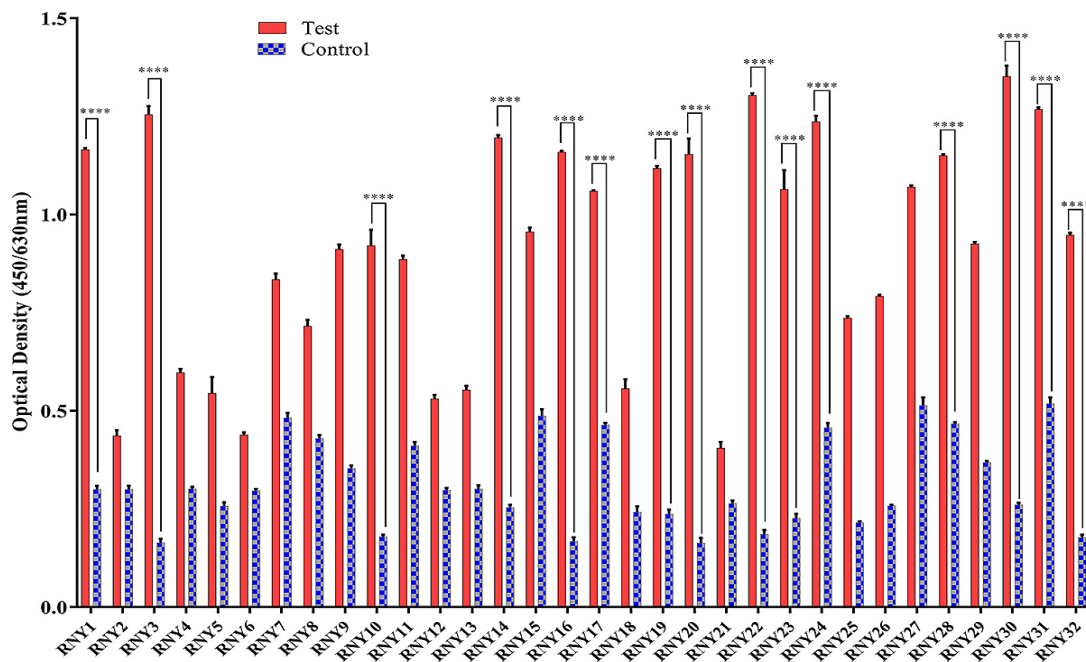


Figure 2. Interaction between 32 individual clones and NTRK2. The specificities and affinities of phage-displayed peptides were detected for NTRK2 (red) and human serum polyclonal antibodies (blue) as a negative control. Individual peptides demonstrated a higher affinity for the target protein compared to the control. Mean \pm SD values of absorbance (450nm) are plotted for each plagues as three independent determinations. Unpaired t-test, 4 df, $p < 0.0001$.

revealed different antigen binding abilities. Eventually, only fifteen strongly positive

phage plaques were selected for sequencing and further study.

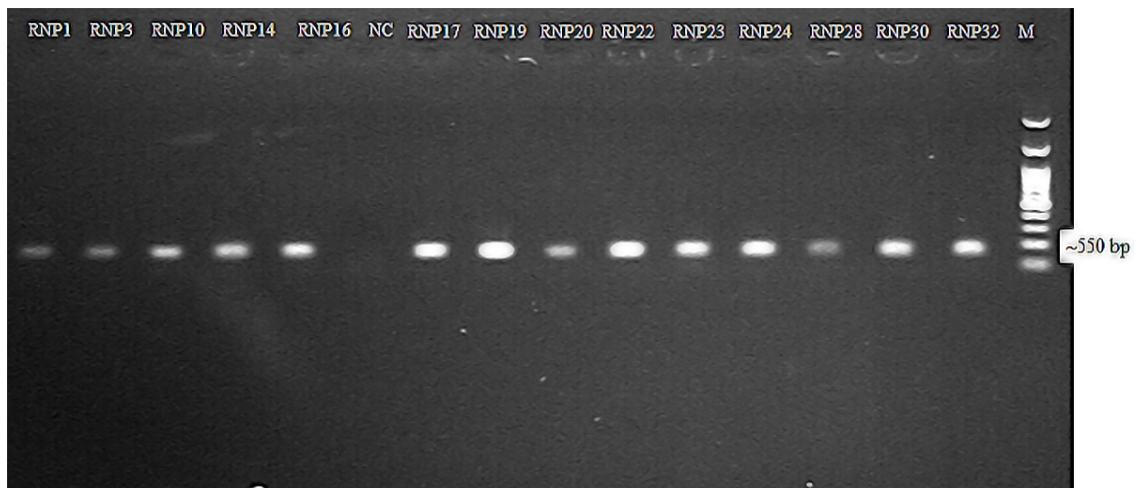


Figure 3. Phage DNA fragment contain nucleotide sequence of displayed peptides (550bp). Lanes from left to right: RNP clones 1, 3, 10, 14, 16, NC (negative Control without DNA template), 17, 19, 20, 22, 23, 24, 28, 30, 32 and M as a 250-bp marker.

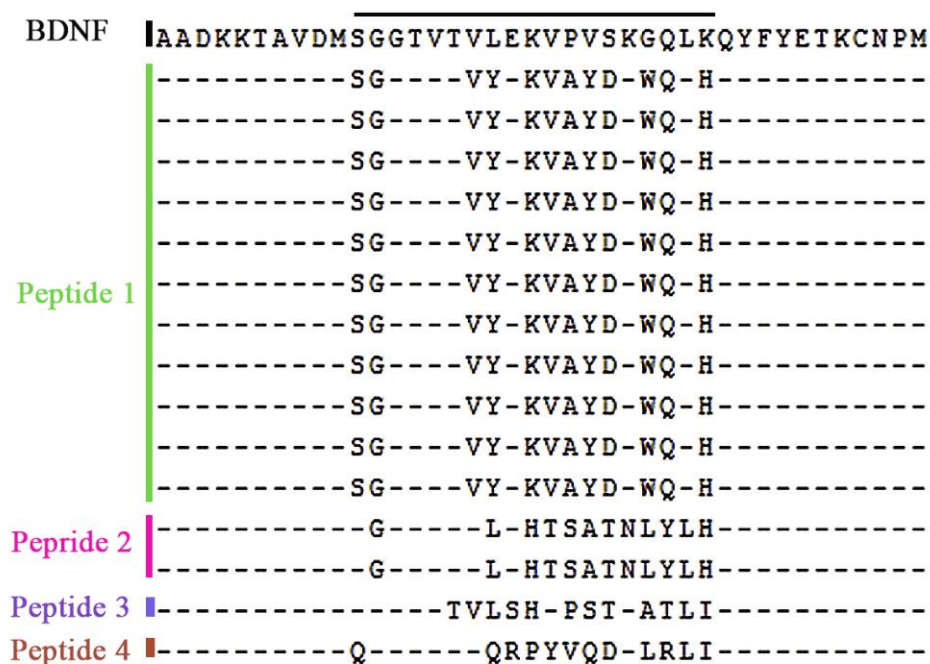


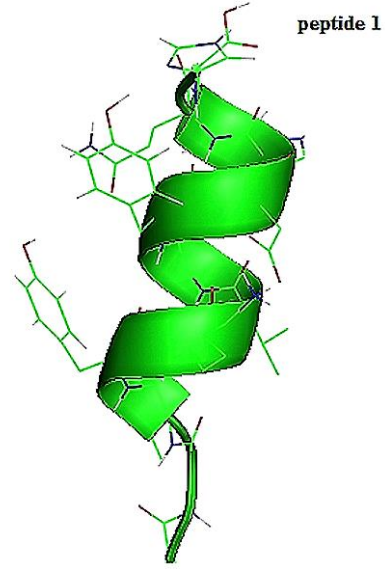
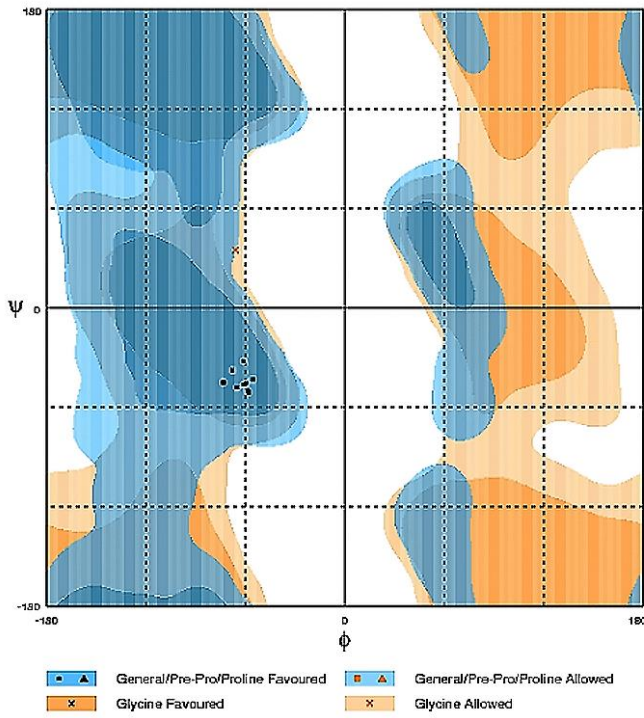
Figure 4. BDNF-mimetic peptides sequences that selected against NTRK2. The peptide sequences were multiple-aligned and showed similarity to the NTRK2 binding region on human BDNF (labeled with a double line comprising the epitope as defined by overlapping peptide analysis).

In the next step, four individual sequences were obtained from strongly positive clones.

The sequence of clones 1, 10, 16, 19, 22, 23, 24, 28, 30, 31, and 32 was

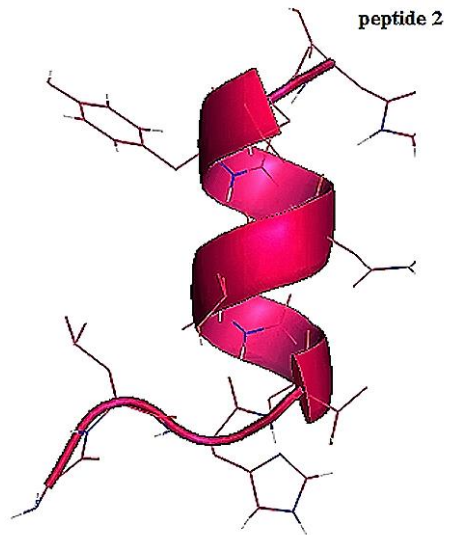
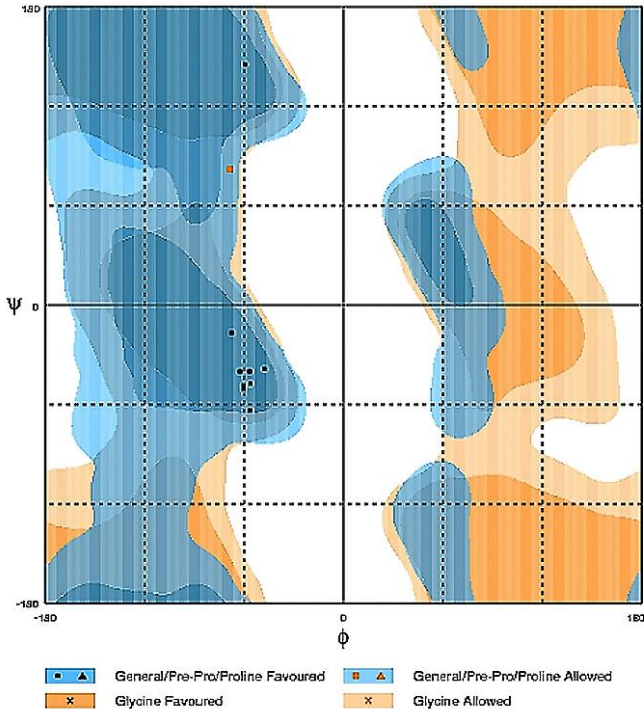
SGVYKVAYDWQH (peptide 1); the sequence of clones 3 and 14 was

GLHTSATNLYLH (peptide 2); the sequence of clone 17 was TVLSHPSTATLI (peptide 3);



1 S G V ■ K V A ■ D ■ Q H 12

Number of residues in favoured region (~98.0% expected) : 9 (90.0%)
 Number of residues in allowed region (~2.0% expected) : 1 (10.0%)
 Number of residues in outlier region : 0 (0.0%)



1 G L H T S ■ T N L Y L H 12

Number of residues in favoured region (~98.0% expected) : 9 (90.0%)
 Number of residues in allowed region (~2.0% expected) : 1 (10.0%)
 Number of residues in outlier region : 0 (0.0%)

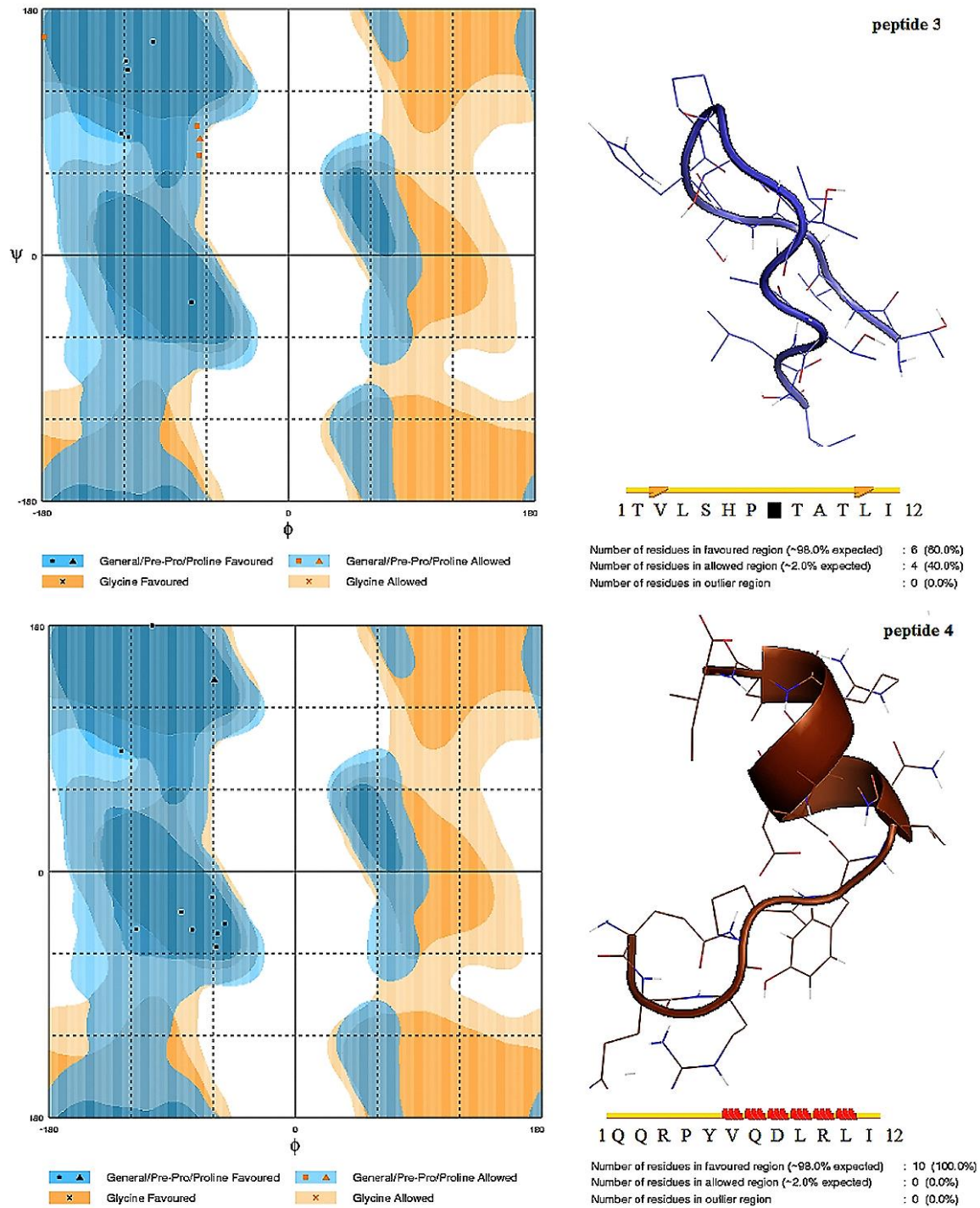


Figure 5. Modeled patterns of the most stable peptide's structures. The peptides were modeled *ab initio* by PEP-FOLD and visualized using PyMOL software. The alpha-helix (H), turn (T) and isolated β bridge (B) of secondary structures are also shown in red, yellow and orange, respectively. The conformational clusters correspond to each peptide identified from the Ramachandran ($\phi\phi$) plot for helix, β turn and bridge conformations. The Ramachandran Plots showed no residues in non-allowed regions for these structures.

and the sequence of clone 20 was QQRPYVQDLRLI (peptide 4). The alignment

of these sequences and BDNF showed that the resulting peptides conformationally mimicked

loop 2 (E40-KVPVSKGQLK-Q51) of BDNF (Figure 4). These conserved residues in BDNF consist of Ser32 (aligned with Ser1 of peptide 1), Gly33 (with Gly2 of peptide 1 and Gly1 of peptide 2), Thr37 (with Thr1 of peptide 3), Val38 (with Val 3 of peptide 1 and Val 2 peptide 3), Leu39 (with Leu2 of peptide 2 and Leu3 of peptide 3), Lys41 (with Lys5 of peptide 1), Val42 (with Val6 of peptide 1), Pro43 (with Pro6 of peptide 3), Val44 (with Val6 of peptide 4), Gln48 (with Gln11 of peptide 1), Leu49 (with Leu11 of peptide 2, 3 and 4). In addition, GLAM2SCAN algorithm (<http://meme-suite.org/doc/glam2scan.html>) (24) introduced SGVYKVAYDWQH sequence as a motif due to the most frequency and similarity with loop2. Therefore, this motif could be applied for ligand binding to the receptor on target cells, similar to loop 2 of BDNF.

Peptide modeling and energy minimization were performed by the PEP-FOLD server. For each model generated, up to 10 numbers were reported: avg, gdt, max, q, tm and sOPEP, where gdt is the Global Distance Test (GDT_TS) to quantify prediction, q is the predicted Q mean score and tm is the predicted TM score. However, the cluster ranks were defined according to their sOPEP. The sOPEP is coarse grained energy of PEP-FOLD based on OPEP (Optimized Potential for Efficient structure Prediction) as a sum of local, non-bonded and hydrogen-bond (H-bond) energies. Accordingly, the best cluster for each peptide (Figure 5) had the best scores with the lowest sOPEP energy (respective highest tm value). These energy scores were -14.49, -9.79, -9.44,

and -10.55 correspond to peptide 1, 2, 3, and 4, respectively. Although, the alpha-helix was present in the most stable models (peptide 1, 2 and 4), peptide 1 due to the lowest energy level showed the maximum stability. However, alpha-helices constitute the largest class of protein secondary structures, and play a major role in mediating protein-protein interactions (25). In principle, the helical structure has a low conformational heterogeneity and an high resistance to proteases as these enzymes typically bind their substrates in the extended conformation (26). That's why, all clusters were submitted to Protein Model Data Base (peptides 1, 2, 3 and 4 as PMDB ID: PM0081104, PM0081105, PM0081106, PM0081107, respectively) and suggested as BDNF peptidomimetics.

4. Conclusion

The 12-meric peptide sequences predicted by the phages imitated a discontinuous epitope of the BDNF-loop 2 which includes critical binding residues. Ibanez and Ilag previously indicated that loop 2 was responsible for specific receptor binding and biological activity (27-29) of BDNF. Likewise, O'Leary and Hughes designed and synthesized multiple peptides based on this region and demonstrated that designed bicyclic and tricyclic dimeric peptides improved neuronal survival in culture environment (17, 30, 31). We suggest that loop 2-mimic peptides may behave like partial BDNF agonists [32]. However, there are still some interesting questions to be answered about the details of these molecules. The structural and functional

relationships and the desirable pharmacological dose for receptor activation should be investigated. Our aim will be at generating novel classes of NTRK2 agonists that may have a potential in neuroprotective approaches.

Acknowledgment

We are grateful to the Department of Biotechnology of Tarbiat Modares University, and Ophthalmic Research Center of Shahid Beheshti University for providing financial support. We are thankful to Dr S Gharavi (Alzahra University, Tehran) and Dr N K Tafreshi (Moffitt Cancer Center and Research Institute, Florida) for technical support.

Reference

- [1] Koshimizu H, et al. Multiple functions of precursor BDNF to CNS neurons: negative regulation of neurite growth, spine formation and cell survival. *Mol. Brain* (2009)2(1):1.
- [2] Nagahara AH, Tuszynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. *Nat. Rev. Drug Discov.* (2011)10(3):209-219.
- [3] Costa A, Peppe A, Carlesimo GA, Zabberoni S, Scalici F, Caltagirone C, Angelucci F. Brain-derived neurotrophic factor serum levels correlate with cognitive performance in Parkinson's disease patients with mild cognitive impairment. *Front. Behav. Neurosci.* (2015)9(1):1-8.
- [4] Ventriglia M, et al. Serum brain-derived neurotrophic factor levels in different neurological diseases. *BIOMED. RES. INT.* (2013)2013(1):1-7.
- [5] Budni J, Bellettini-Santos T, Mina F, Garcez ML, Zugno AI. The involvement of BDNF, NGF and GDNF in aging and Alzheimer's disease. *Aging Dis.* (2015)6(5):331-341.
- [6] Ihara K, et al. Serum BDNF levels before and after the development of mood disorders: a case-control study in a population cohort. *Transl. Psychiatry* (2016)6(4):782-789.
- [7] Ma X-c, et al. Intranasal Delivery of Recombinant AAV Containing BDNF Fused with HA2TAT: a Potential Promising Therapy Strategy for Major Depressive Disorder. *Sci. Rep.* (2016)6(22):404-413.
- [8] Fyfe I. Movement disorders: Targeted RNA or BDNF gene transfer protects against frataxin deficiency. *Nature Reviews Neurology.* (2016)19.
- [9] Nagahara AH, et al. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat. Med.* (2009)15(3):331-337.
- [10] Tuszynski MH, Nagahara AH. NGF and BDNF Gene Therapy for Alzheimer's Disease. *Transl. Neurosci.* (2016)2016(1):33-64.
- [11] White AO, et al. BDNF rescues BAF53b-dependent synaptic plasticity and cocaine-associated memory in the nucleus accumbens. *Nat. Commun.* (2016)7(117):25-37.
- [12] Matsuoka Y, et al. Serum pro-BDNF/BDNF as a treatment biomarker for response to docosahexaenoic acid in traumatized people vulnerable to developing psychological distress: a randomized controlled trial. *Transl. Psychiatry.* (2015)5(7):596-602.
- [13] Xue W, et al. PKA-CREB-BDNF signaling regulated long lasting antidepressant activities of Yueju but not ketamine. *Sci Rep.* (2016)6:26331.
- [14] Kozisek ME, Middlemas D, Bylund DB. Brain-derived neurotrophic factor and its receptor tropomyosin-related kinase B in the mechanism of action of antidepressant therapies. *Pharmacol Ther.* (2008)117(1):30-51.
- [15] Song J-H, Yu J-T, Tan L. Brain-derived neurotrophic factor in Alzheimer's disease: risk, mechanisms, and therapy. *Mol. Neurobiol.* (2015)52(14):77-93.
- [16] Novkovic T, Mittmann T, Manahan-Vaughan D. BDNF contributes to the facilitation of

- hippocampal synaptic plasticity and learning enabled by environmental enrichment. *Hippocampus* (2015)25(1):1-15.
- [17] O'Leary PD, Hughes RA. Design of potent peptide mimetics of brain-derived neurotrophic factor. *J. Biol. Chem.* (2003)278(257):38-44.
- [18] Massa SM, et al. Small molecule BDNF mimetics activate TrkB signaling and prevent neuronal degeneration in rodents. *J. Clin. Invest.* (2010)120(17):74-85.
- [19] Longo FM, Xie Y, Massa SM. Neurotrophin small molecule mimetics: candidate therapeutic agents for neurological disorders. *Cent. Nerv. Syst. Agents Med. Chem.* (2005)5(1):29-41.
- [20] Katsu-Jiménez Y, Loría F, Corona JC, Díaz-Nido J. Gene Transfer of Brain-derived Neurotrophic Factor (BDNF) Prevents Neurodegeneration Triggered by FXN Deficiency. *Mol. Ther.* (2016)24(5):877-889.
- [21] Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* (2013)30(12):2725-2729.
- [22] Lamiable A, Thévenet P, Rey J, Vavrusa M, Derreumaux P, Tufféry P. PEP-FOLD3: faster de novo structure prediction for linear peptides in solution and in complex. *Nucleic Acids Res.* (2016)2016(1):449-454.
- [23] Maupetit J, Derreumaux P, Tufféry P. A fast method for large scale De Novo peptide and miniprotein structure prediction. *J. Comput. Chem.* (2010)31(4):726-738.
- [24] Frith MC, Saunders NF, Kobe B, Bailey TL. Discovering sequence motifs with arbitrary insertions and deletions. *PLoS Comput Biol.* (2008)4(4):1-12.
- [25] Guharoy M, Chakrabarti P. Secondary structure based analysis and classification of biological interfaces: identification of binding motifs in protein-protein interactions. *Bioinformatics.* (2007)23(15):1909-1918.
- [26] Tyndall JD, Nall T, Fairlie DP. Proteases universally recognize beta strands in their active sites. *Chem. Rev.* (2005)105(3):973-1000.
- [27] Ibanez C, Ebendal T, Persson H. Chimeric molecules with multiple neurotrophic activities reveal structural elements determining the specificities of NGF and BDNF. *The EMBO journal.* (1991)10(8):2105-2110.
- [28] Ibanez C, Ilag L, Murray-Rust J, Persson H. An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. *EMBO J.* (1993)12(6):2281.
- [29] Ilag LL, Lönnerberg P, Persson H, Ibáñez CF. Role of variable beta-hairpin loop in determining biological specificities in neurotrophin family. *J. Biol. Chem.* (1994)269(31):19941-19946.
- [30] O'Leary P, Hughes R. Structure-Activity Relationships of Conformationally Constrained Peptide Analogues of Loop 2 of Brain-Derived Neurotrophic Factor. *J. Neurochem.* (1998)70(4):1712-1721.
- [31] Fletcher JM, Hughes RA. Novel monocyclic and bicyclic loop mimetics of brain derived neurotrophic factor. *J. Pept. Sci.* (2006)12(8):515-524.
- [32] Nafian F, Rasaei M, Yazdani S, Daftarian N, Soheili Z, Kamali Doust Azad B. Peptide selected by phage display increases survival of SH-SY5Y neurons comparable to brain-derived neurotrophic factor. *J. Cell Biochem.* 2018;120(3):1-11.

ONLINE SUBMISSION

www.ijps.ir