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RESEARCH ARTICLE

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Development of α4 integrin DNA aptamer as a potential therapeutic tool for multiple sclerosis

Shirin Kouhpayeh[1] | Zahra Hejazi[2] | Maryam Boshtam[3] | Mina Mirian[4] | Ilnaz Rahimmanesh[2] | Leila Darzi[5] | Abbas Rezaei[6] | Laleh Shariati[7,8] | Hossein Khanahmad[2]

[1]Department of Immunology, Erythron Pathobiology and Genetics Laboratory, Isfahan, Islamic Republic of Iran
[2]Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[3]Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[4]Department of Pharmaceutical Biotechnology, Isfahan Pharmaceutical Science Research Center, School of Pharmacy and Pharmaceutical Science, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[5]Department of Medical Biotechnology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Islamic Republic of Iran
[6]Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[7]Applied Physiology Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[8]Department of Biomaterials, Nanotechnology and Tissue Engineering, School of Advanced Technologies in Medicine, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran

Correspondence
Hossein Khanahmad, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan 8174673461, Iran.
Email: H_khanahmad@med.mui.ac.ir

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Abstract

One of the most important molecules for multiple sclerosis pathogenesis is α4 integrin, which is responsible for autoreactive leukocytes migration into the brain. The monoclonal antibody, natalizumab, was introduced to market for blocking the extravasation of autoreactive leukocytes via inhibition of α4 integrin. However, the disadvantages of antibodies provided a suitable background for other agents to be replaced with antibodies. Considering the profound advantages of aptamers over antibodies, aptamer isolation against α4 integrin was intended in the current study. The α4 integrin-specific aptamers were selected using cell-systematic evolution of ligands by exponential enrichment (SELEX) method with human embryonic kidney (HEK)-293T overexpressing α4 integrin and HEK-293T as target and control cells, respectively. Evaluation of selected aptamer was performed through flow cytometric analysis. The selected clones were then sequenced and analyzed for any possible secondary structure and affinity. The results of this study led to isolation of 13 different single-stranded DNA clones in 11 rounds of selection which were categorized to three clusters based on common structural motifs and the equilibrium dissociation constant (K_d) of the most stable structure was calculated. The evaluation of SELEX progress showed growth in aptamer affinity with increasing of the number of cycles. Taken together, the findings of this study demonstrated the isolation of α4-specific single-stranded DNA aptamers with suitable affinity for ligand, which can further be replaced with natalizumab.

KEYWORDS

aptamer, integrin α4, multiple sclerosis, systematic evolution of ligands by exponential enrichment aptamer technique

1 | INTRODUCTION

Integrins are a family of glycoprotein adhesion molecules with a heterodimeric transmembrane structure which comprise an α and a β subunits (approximately 1000 and 800 amino acids, respectively). The structural and functional diversity of integrins enable this family of adhesion molecules to play a pivotal role in a broad range of biological functions, including development, immune responses, leukocyte trafficking, hemostasis, and cancer.[1] α4 Integrins such as α4β1 and α4β7 participate in leukocyte recruitment through rolling adhesion before activation and firm adhesion after activation in the brain and gut endothelium, respectively.[2]

Studies in animal models targeting α4β7 with an antibody validated the role of this adhesion molecule in ulcerative colitis and Crohn's disease (CD).[3] Additionally, the evaluation of small-molecule antagonists designed against α4β7 and dual-acting agents against α4β1 and α4β7 illustrated the blockade of both α4β1 and α4β7 to attach the corresponding ligands, thus inhibiting subsequent leukocyte recruitment in multiple sclerosis (MS) and CD.[4]

The most prominent manifestation of MS is the plaque formation. Various elements are important to produce demyelinated plaques, including autoantibodies, cytokines, and activated autoreactive CD4+ T cells, which play the most crucial role in this process.[5] The migration of autoreactive immune cells through the blood-brain barrier, is considered as the pathologic hallmark of MS.[6] Based on the importance of α4 integrin in MS pathogenesis, natalizumab (Tysabri), a blocking antibody to α4 integrin was approved for treatment of patients with relapsing MS.[7-9]

There are some novel therapeutic tools that are capable of target therapy in different context.[10-13] Aptamers as one of those therapeutic tools are single-stranded DNA (ssDNA) or RNA molecules that are typically less than 100-mer, which have the ability to bind to a wide range of targets, with high affinity and specificity.[14,15] The advantages of aptamers over antibodies consist of high stability, production, low immunogenicity and variety of targets.[16] Taken together, aptamers make a powerful tool in the diagnosis and treatment of diseases as well as by employing aptamers as biosensors. Thereby, aptamers are considered to be an alternative to antibodies in many biological and nonbiological applications.[17]

Aptamers are evolved from random oligonucleotide pools by a process called the systematic evolution of ligands by exponential enrichment (SELEX).[18,19] The evolution of aptamers is possible due to the ability of these small oligonucleotides to fold into unique three-dimensional (3D) structures that can interact with the target of interest with high specificity and affinity.[20]

As all the therapeutic approaches for MS were designed based on the disease pathogenesis, and also the advantages of aptamers over antibodies. This study was conducted to replace natalizumab, the first line therapy for MS, with aptamers which are targeted against the α4 integrin, using the cell-SELEX method with the proteins immobilized on the cell surface.

2 | MATERIALS AND METHODS

2.1 | Library and primers

A DNA library and primers were ordered to Tag Copenhagen A/S (Frederiksberg, Denmark). An 88-bp oligonucleotide single-stranded DNA (ssDNA) library, consisting of a 52-bp randomized region flanked on either side by an 18-bp primer hybridization site was used to generate aptamers against α4 integrin. This library contained more than 10^{15} unique ssDNA.

2.2 | Target and control cells

The target cells, human embryonic kidney 293T (HEK-293T) overexpressing α4 integrin, ITGA4-HEK, was constructed in previous research,[21] and HEK-293T (Pasteur Institute of Iran, Tehran, Iran) served as control cells.

2.3 | Cell culture

The ITGA4-HEK and HEK-293T cell lines, were maintained in media containing 90% high-glucose Dulbecco's modified Eagle's medium (Bioidea, Tehran, Iran) with 4.0 mM L-glutamine supplemented with 10% fetal bovine serum (Bioidea). The cells were passaged every 3 to 4 days or once they reached 90% confluency and grown in 5% CO_2 at 37℃.

2.4 | Cell SELEX

DNA library (0.5 mM) was resuspended in binding buffer (Dulbecco's phosphate-buffered saline [DPBS], $MgCl_2$, glucose, transfer RNA [tRNA], and bovine serum albumin), mixed and heated at 95°C for 5 minutes, and then snap-cooled on ice until the cell-SELEX process. For the first round of selection, 1×10^6 ITGA4-HEK cells, were used for collecting specific sequences. The snap-cooled DNA library was added to the cell suspension and incubated for 1 hour at 4°C. The cell pellet was then resuspended in binding buffer heated at 95°C for 10 minutes, centrifuged at 13 100*g* for 5 minutes, and the eluted ssDNA was collected.

At least 1×10^6 HEK-293T cells were used for negative selection. The cells were washed with washing buffer

TABLE 1 The stringency changes during the SELEX process

	Library concentration (nmol)	Cell count	tRNA (%wt/vol)	Incubation time (min)	Mg^{2+} (%vol/vol)
Round 1	10	10^7	0.01	60 min	0.5
Round 3	5	2.5×10^6	–	40	–
Round 7	2.5	10^6	–	30	–
Round 11	1.25	–	0.02	20	0.25

Abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; tRNA, transfer RNA.

(DPBS, $MgCl_2$, and glucose) and incubated with the eluted DNA pool from positive selection on ice for 1 hour. After incubation, the cells containing the binding sequences were spun down at 3059*g* for 5 minutes. The supernatant was recovered containing sequences that do not bind to control cells.

The selected pool in each round of SELEX at 10% (vol/vol) in binding buffer, serves as the template to optimize the number of cycles for polymerase chain reaction (PCR) procedure. The PCR amplification was performed using forward and reverse primers (5′-ATACCAGCTTATT CAATT-3′, 5′-AGATTGCACTTACTATCT-3′, respectively). The amplification condition was divided to three steps of 95℃ (150 seconds), 4, 6, 8, 10, 12 or 16 cycles of 95℃ (30 seconds), 42℃ (30 seconds), and 72℃ (30 seconds), with a final step of 72℃ (150 seconds).

Agarose gel electrophoresis was used to determine a bright band without nonspecific amplicons. The cycle number of interest was selected and PCR amplification performed to yield 1000 μL of sublibrary containing the ssDNA required for the next round of SELEX. For producing the sublibrary, phosphate-conjugated reverse primer, was used for further digestion of the lagging strand, using λ-exonuclease III (Thermo Fisher Scientific, Waltham, MA).

The PCR product was treated with λ-exonuclease III as described earlier,[22] dried and diluted in binding buffer, to yield a final concentration of 5 nM for the sublibrary.

The positive selection cycle was repeated 11 times, along with counter selection, with a slight increase in annealing temperature to 45℃. The stringency of selection was gradually increased by changing concentration of library, tRNA and $MgCl_2$, cell count and incubation time (Table 1).

2.5 | Monitoring of ssDNA generation

The first ssDNA library was used as a template for quantitative real-time PCR as explained previously.[22] In brief, real-time PCR was used for amplification of the initial library, followed by the enzymatic digestion of double stranded DNA in one of the sets of triplicate. Subsequently, the melting curve analysis was carried out, to evaluate the ssDNA generation from double stranded DNA. Alterations in the intensity of the fluorescence was monitored constantly and melting temperatures (Tms) were calculated with the "StepOnePlus" software (Version 2.2; StepOnePlus Real-Time PCR; Life Technology, Carlsbad, CA).

To monitor the ssDNA generation in final rounds, different concentrations of the 11th selected pool, were loaded on 12% nondenaturing polyacrylamide gel electrophoresis, in 1% tris-borate-EDTA buffer. Samples were denatured by heat treatment at 95°C for 5 minutes and then snap-cooled on ice. The electrophoresis was carried out at 4°C, using 70 V for 2 hours, after prerunning the gel for 10 minutes with 90 V. The gel was stained with silver-nitrate for visualization.

2.6 | SELEX monitoring

To screen the DNA molecules selected after cell-SELEX, a flow cytometric assay was performed to determine the ability of the fluorescein isothiocyanate (FITC)-conjugated DNA molecules to bind selectively to the α4 integrin expressing HEK-293T cells.

The PCR program was performed as described previously using FITC-conjugated forward and 5′-phosphate reverse primers. The products were then subjected to λ-exonuclease III digestion. A total of 50 μL of the ssDNA selected pool was added to each tube containing 5×10^5 ITGA4-HEK cells resuspended in binding buffer. Unselected DNA library was served as the negative control pool. The mixture was incubated on ice for 30 minutes, washed three times using washing buffer, and then resuspended in 200 μL of binding buffer. Flow cytometric analysis was performed using BD FACSCallibur (BD Bioscience, San Jose, CA), and the data analyzed by FlowJo data analysis software package (version 7.6.1; TreeStar, Ashland, Oregon). Subsequently, the equilibrium dissociation constants (K_d) was calculated using equation $Y = B_{max}\ X/(K_d + X)$, where X is the aptamer concentration, Y is the mean fluorescence intensity (MFI) of each aptamer, and B_{max} is the maximum MFI using SigmaPlot software (Version 12.3; Jandel, San Rafael, CA).

2.7 | Clustering and prediction

The PCR amplification was performed using unconjugated primers and the PCR product was cloned using a TA

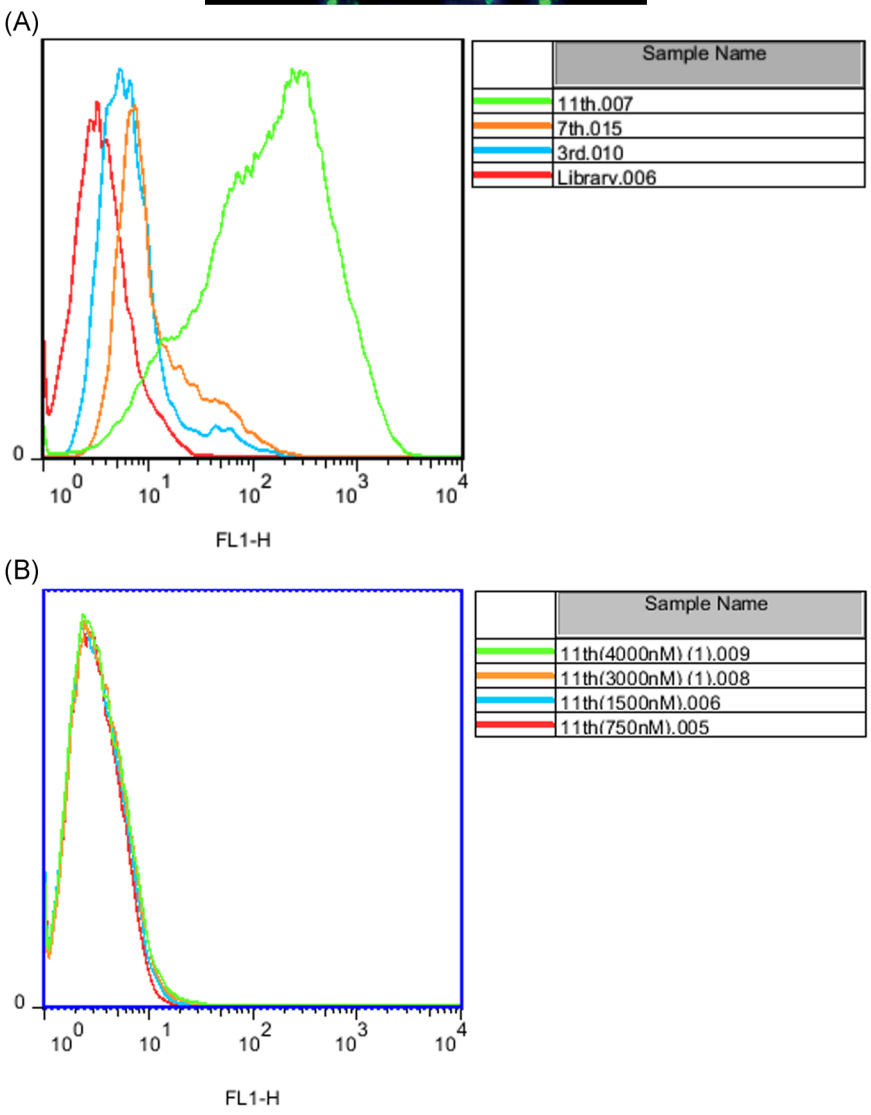

FIGURE 1 Cell-SELEX progression monitoring. Cell samples were prepared for the flow cytometry aptamer binding assay with 1×10^5 cells and 750 nM of DNA aptamers. FITC fluorescence emission of the live cell population is presented for the: (A) initial library (B) third (C) seventh, and (D) eleventh rounds of SELEX which have the mean fluorescent intensity, 6%, 18.8%, and 79.3%, respectively. FITC, fluorescein isothiocyanate; SELEX, systematic evolution of ligands by exponential enrichment

cloning vector (SinaClon, Tehran, Iran). The positive clones were selected, amplified and sequenced (Noor Genetic Lab, Ahwaz, Iran). The sequencing data were analyzed using Chromas software (Version 1.45, http://www.technelysium.com.au/chromas.html, South Brisbane, Australia). The sequence was aligned using ClustalX software (Version 2.1, http://www.clustal.org, Dublin, Ireland) and clustered based on common motifs. The most thermodynamically stable secondary structures were chosen using DNAMAN software (Version 7.0; The RNA Institute, New York, NY).

3 | RESULTS

3.1 | PCR optimization

In each round of SELEX procedure, PCR optimization showed the best number of cycles, without any amplicons, which were between 8 and 14 cycles for the first to the 11th selected pools.

3.2 | Monitoring the progress of selection

The initial library was used as negative control (Figure 1A). The fluorescent intensity of FITC was improved with increasing the number of SELEX rounds (Figure 1B-D). No fluorescent emission was detected from cells that received 750 nM of initial library, while fluorescent intensity increased with using 750 nM of the third, seventh, and eleventh rounds of SELEX to 6%, 18.8%, and 79.3%.

3.3 | Clustering and prediction

The α4 integrin aptamer candidates were presented in three main clusters of the phylogenetic tree, based on sequence identity (Figure 2A). The alignment and grouping indicated three families of aptamer candidates using ClustalX 2.1 for which the shared random sequence

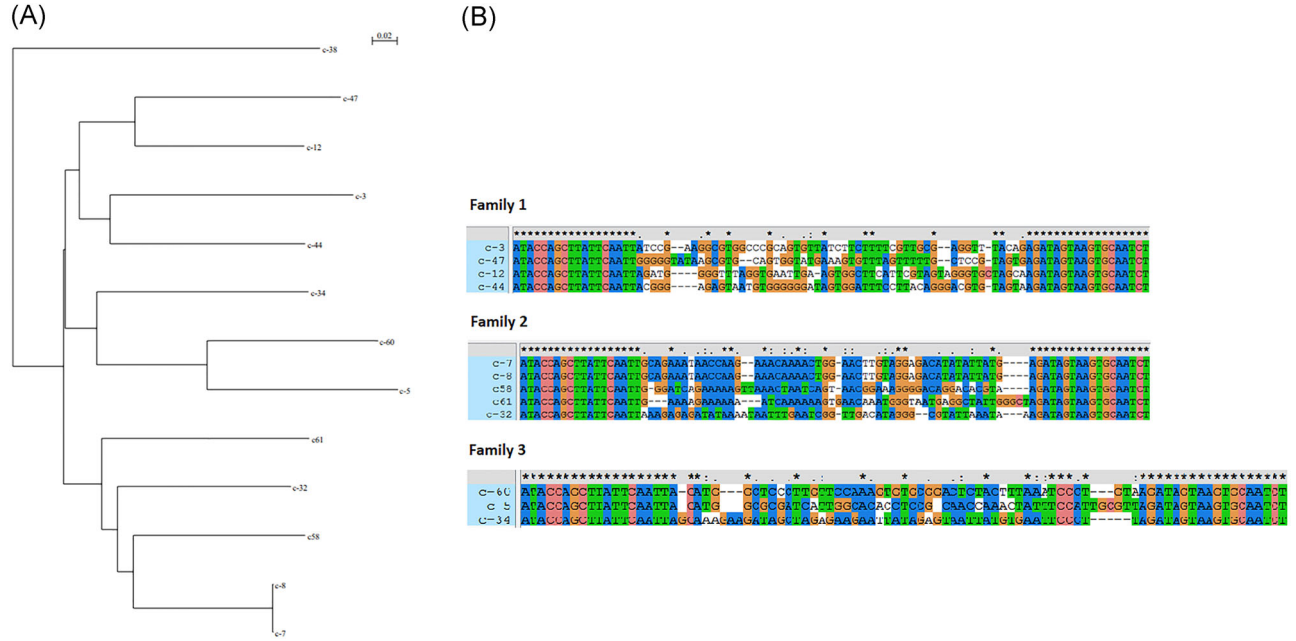

FIGURE 2 Phylogenetic tree, alignment and clustering. A, ClustalX phylogenetic tree analysis of the 88 bp DNA sequences from 13 different clones. B, DNA sequence alignment. ClustalX alignment of the 88 bp sequences for the DNA sequences in the 11th SELEX round pool illustrated three families of sequences emerge that have 90% sequence identity within each family. Common nucleotides in all families are denoted with an asterisk. SELEX, systematic evolution of ligands by exponential enrichment

motif identity was over 90% within each family (Figure 2B and Table 2).

The lowest potential energy structures for each 88 bp-aptamer candidate, were generated by DNAMAN software (Figure 3C). All three aptamer candidates form the lowest free energy secondary structures with a large open loop and three to six small stem loop motifs. Determination of K_d for the most stable aptamer (C-12) with the highest affinity was 215.36 ± 0.66 nM which is presented in Figure 4.

4 | DISCUSSION

Aptamers as a novel active agent was used against α4 integrin in this study due to ability of folding into a 3D structure[23] and discriminating related targets that have characteristics in common.[17]

Isolation of aptamer against adhesion molecules was performed using recombinant proteins of interest in previous studies.[24-27] Although using recombinant proteins led to the internalization of aptamer during SELEX

TABLE 2 Aptamer sequences

Clone	Sequence	Family
C-3	ATCCGAAGGCGTGGCCCGCAGTGTTATCTTCTTTTCGTTGCGAGGTTTACAG	1
C-12	AGATGGGGTTTAGGTGAATTGAAGTGGCTTCATTCGTAGTAGGGTGCTAGCA	1
C-44	ACGGGAGAGTAATGTGGGGGGATAGTGGATTTCCTTACAGGGACGTGTAGTA	1
C-47	GGGGGTATAAGCGTGCAGTGGTATGAAAGTGTTTAGTTTTTGCTCCGTAGTG	1
C-7	GCAGAAATAACCAAGNAAACAAAACTGGAACTTGTAGGAGACATATATTATG	2
C-8	GCAGAAATAACCAAGNAAACAAAACTGGAACTTGTAGGAGACATATATTATG	2
C-58	GGGATCAGAAAAAGTTAAACTAATCAGTAACGGAAAGGGGACAGGACACGTA	2
C-61	GAAAAGAAAAAAATCAAAAAAGTGAACAAATGGGTAATGAGGCTATTGGGCT	2
C-32	AAAGAGAGATATAAAATAATTTGAATCGGTTGACATAGGGCGTATTAAATAA	2
C-34	AGCANAGAAGANAGCTAGAGAAGAATTATAGAGTAATTATGTGAATTCCCTT	3
C-60	ACATGGCTCCCTTGTTCCAAAGTGTGCGGACTCTACTTTAAATCCCTGTA	3
C-5	ACATGGCGCGATCATTGGCACACCTCCGCAACCAAACTATTTCCATTGCGTT	3
C-38	AGATAGTAAGTGAGATTGCACTTACTATCTTGAGTTCACTTATACCCAATTG	Unclustered

Note. The fixed regions for the primer binding site are deleted and the α4 integrin-specific aptamer sequences are listed.

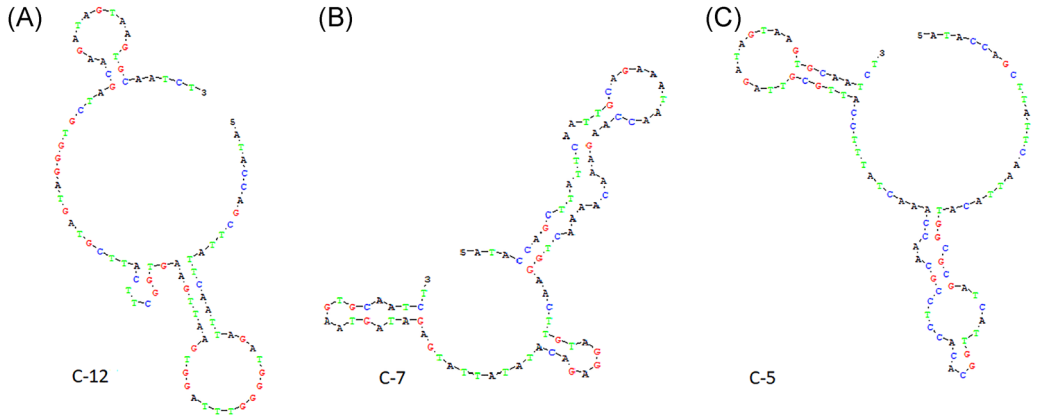

FIGURE 3 Secondary structure predictions. Predicted secondary structures for the three families of aptamer candidates that were chosen from the 11th round of SELEX. (A) to (C) are representatives of families 1 to 3, respectively with lowest potential energy structures in each family to bind α4 integrin expressing cells. Secondary structures were predicted by DNAMAN software. SELEX, systematic evolution of ligands by exponential enrichment

procedure.[28] Moreover, commercial recombinant proteins are rarely available in full length. The recombinant form of α4 integrin, contains the motif, which is involved in cell adhesion. Thus, it may not fold to the same conformation as the surface expressed receptor. A recent study by Chenna et al[29] has shown that stoichiometric differences between the natural receptor and the recombinant isoform, may influence the final outcome in therapeutic studies. Herein, α4 integrin overexpressing cells were used as a platform for specific aptamers partitioning. In a similar study, Kim et al[30] produced a HepG2 cell line overexpressing EpCAM. Eukaryotic proteins, usually have posttranslational modifications (PTMs)[31,32] and can be expressed in prokaryotic expression systems in high yield and low cost. However, these proteins lack PTM and even proper folding.[33] Due to targeting the special epitopes which contain PTM residues by the majority of diagnostic and therapeutic antibodies,[34] displaying the eukaryotic proteins in mammalian cell lines, for example, HEK-293T, is an alternative method to overcome the above cited obstacles.[21,35] In the current study, α4 integrin-specific aptamers were isolated using α4 integrin overexpressing HEK-293T cells in a modified cell-SELEX method with a counter selection step in each round to avoid the selection of nonspecific binders against α4 integrin.

In previous studies, specific oligonucleotide aptamers were isolated using various SELEX methods against different adhesion molecules which amongst, only two studies applied counter selection step.[24,30] The positive selection which was performed routinely alone, leads to

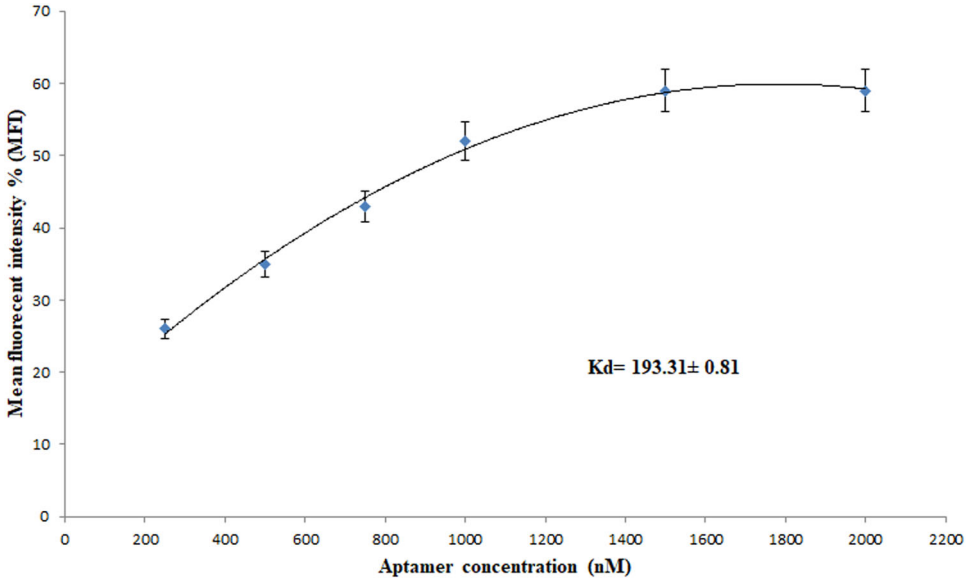

FIGURE 4 K_d determination. The selected individual aptamer (C-12) K_d was calculated using SigmaPlot software in various concentrations. Data are presented as mean ± SD. K_d, dissociation constant

the isolation of specific and nonspecific complexes of oligonucleotides with low affinity (500-1000 nM).[25-27]

Considering the crucial role of ssDNA in SELEX procedure, monitoring of ssDNA generation is of great importance. Our previous study has shown for the first time, that melting curve analysis using real-time PCR may serve as a reliable method for the visualization of ssDNA generation from the PCR amplified products, particularly in the initial rounds of SELEX.[22] Previous studies have also applied real-time PCR for assessment of aptamer complexity and pool size based on half-renaturation time.[36,37]

Affinity monitoring of selected aptamer, in our study, was performed using flow cytometric analysis to verify the affinity growth. There are several affinity monitoring procedures including electrophoresis mobility shift assay, radioassay, real-time PCR, confocal microscopy, and FACS analysis, which are designed based on SELEX method.[25,27,30,38-40] Compared to quantitative methods, for example, flow cytometric analysis, some of the designations are only qualitative methods, which are incapable of proper monitoring.[39]

The isolated α4 integrin aptamer candidates in this study were presented in three main clusters based on sequence identity. The lowest potential energy (ΔG) structures consist of a large open loop and three to six small stem loop motifs. The similarity in these structures illustrates a possible secondary structure requirement for aptamers to bind to α4 integrin. These data are in good accordance with previous studies which the isolated aptamers in different families had different motifs in common.[41] The most stable individual aptamer showed the dissociation constant of 215.36 ± 0.66 nM, which prove the high affinity of this molecule for α4 integrin targeting. The K_d value of the selected aptamer is higher than EpCAM-specific aptamer (5.48 ± 0.84 μM)[30] and less than α6β4 (137 nM)[24] and E- and P-selectin (100 nM)[27] that might be due to the difference between the applied SELEX strategies and affinity monitoring method of choice.

In conclusion, we selected specific aptamers with the proper affinity against α4 integrin which could further be applied as a therapeutic approach for natalizumab replacement in MS therapy. Although the isolated aptamers still need to be tailored for higher stability to employ in experimental autoimmune encephalitis model. Moreover, the efficiency of the selected sequences for restricting the plaque formation should be monitored.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

AUTHOR CONTRIBUTIONS

SK and HK made substantial contributions to conception and design, acquisition of data, analysis, and interpretation of data. LS, MB, MM, and IR involved in drafting the manuscript or revising it critically for important intellectual content. LD involved in acquisition and analysis of data. AR gave final approval of the version to be published. All the authors participated sufficiently in the work, took responsibility for appropriate portions of the content. They agreed to be accountable for all aspects of the work and also ensured that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

ORCID

Maryam Boshtam http://orcid.org/0000-0003-0946-3354

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