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Bioorganic & Medicinal Chemistry Letters 17 (2007) 6119–6122
Bioorganic & Medicinal Chemistry Letters

Binding of an aptamer to the N-terminal fragment of VCAM-1

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Received 26 July 2007; revised 10 September 2007; accepted 11 September 2007
Available online 15 September 2007

Abstract—In vitro selection of 2′-fluoropyrimidine oligonucleotide aptamers was performed against the N-terminal two-domain fragment of mouse VCAM-1. The SELEX procedure enriched the starting pool in a family of homologous sequences. High binding affinity (10 nM) of one member of this family, aptamer 12.11, was demonstrated in a filter binding assay.

Vascular cell adhesion molecule-1 (VCAM-1; CD106) is a member of the immunoglobulin-like superfamily (IgSF) that shows increased expression in a number of pathologic conditions, including tumor metastasis,[1] atherosclerosis,[2,3] allograft rejection,[4] and chronic inflammatory disorders.[5–7] In particular, the interaction with $\alpha_4\beta_1$ integrin (very late antigen [VLA]-4) expressed on leucocytes drives the recruitment of immune cells to inflammation sites, together with the ICAM-1/$\alpha_L\beta_2$ (LFA-1) interaction, another potent pathway in inflammation.[8] An important contribution of the VCAM-1/VLA-4 pathway has been recognized in neuropathologies such as multiple sclerosis (MS)[9] and the relevant animal model experimental allergic encephalomyelitis (EAE),[10] as well as stroke,[11] and ischemia models.[11] In rodents, the basal expression of VCAM-1 was shown to be lower than that of ICAM-1.[12,13] Therefore, VCAM-1 is an attractive endothelial target for brain inflammation monitoring, whether it be chronic or acute.

SELEX (systematic evolution of ligands by exponential enrichment)[14] is a combinatorial strategy enabling the selection of oligonucleotide-based ligands (aptamers[15]) for several kinds of molecular targets, including extracellular proteins.[16] Especially, aptamers have an easy access to intravascular targets, like VCAM-1, which do not require membrane passage. Furthermore, aptamers have been shown in many cases to display an inhibitory activity on their target, depending on the region involved in the binding. Affinity and specificity of aptamers have been reported to be similar to that of antibodies, together with enhanced pharmacokinetics due to their smaller size.[17] We report here the successful selection of an RNA-modified aptamer with high affinity for the N-terminal two-domain fragment of VCAM-1, termed VCAM-1,2d.

pASK-IBA2 vector (IBA GmbH) was used to drive the tetracycline-induced expression of mouse VCAM-1,2d (inserted as a 597 bp BsaI/BsaI fragment) to the bacterial periplasmic space, and to purify the recombinant protein by affinity column (*Strep*®-tag Protein Purification Buffer Set, IBA GmbH), thanks to, respectively, a cleavable N-terminal 21-amino acid signal peptide (ompA[18]) and a non-cleavable C-terminal 10-amino acid tag peptide (*Strep*®-tag II[19]). The expected 23 kDa protein (199 amino acids corresponding to VCAM-1 domains 1 and 2, followed by Strep-Tag II) was successfully expressed by the bacterial strain Rosetta and purified from the periplasmic extract, as assessed by Coomassie blue staining of the SDS–PAGE gel. Azurin (15 kDa) was produced in parallel, as a control, using the same conditions. The maintenance of an

Keywords: SELEX; VCAM-1; Aptamer.
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doi:10.1016/j.bmcl.2007.09.046

intradomain disulfide bridge, critical for a correct immunoglobulin-like folding, was verified by gel migration comparison of reduced versus non-reduced VCAM-1,2d in the presence of an alkylating agent (data not shown).

An initial pool of 87-mer single-strand 2′-fluoropyrimide oligonucleotides (2′F-Py-ODNs), containing a random 40-mer sequence with flanking primer regions of fixed sequences at both ends, was transcribed using $T7^{Y639F}$ RNA polymerase from the corresponding ADN library. This mutated polymerase allowed the incorporation of 2′F-bases prior to selection,[20] so as to yield nuclease-resistant aptamers suitable for in vivo injection.[21]

The SELEX strategy is depicted in Figure 1: Azurin and VCAM-1,2d being immobilized on MPG-Streptactin beads (IBA GmbH) through Strep-Tag II in RPMI medium at 37 °C, a complete round of selection included a negative selection step (counter-selection) against Azurin, after which non-bound sequences were separated by gentle centrifugation, and a positive selection step against VCAM-1,2d, after which bound sequences were recovered by phenol/chloroform extraction and precipitation. Retro-transcription (Superscript II (Invitrogen); primer: TCC TGT TGT GAG CCT CCT GTC GAC; final volume 100 µl), PCR (Accuprime (Invitrogen); forward primer: TAA TAC GAC TCA CTA TAG GGA GAC AAG AAT AAA CGC TCA A; reverse primer: TCC TGT TGT GAG CCT CCT GTC GAC; final volume 1 ml), and transcription ($T7^{Y639F}$ RNA polymerase, final volume 1 ml) of the subsequent DNA pool yielded 2'F-Py-ODNs for the next round. 12 rounds were performed with increasing selection stringency through:

- decreasing the selection time (overnight, 2 h, 1 h, 0.5 h);
- decreasing the quantity of 2′F-Py-ODNs (1.6 to 0.8 nmol);
- increasing the number and duration of washes by RPMI medium (4–8 washes of 2–5 min at the end of the selection step).

The stringency of the selection conditions was designed to progressively increase the competition between aptamers that bind to the target, in order for the selection process to yield high affinity aptamers. In addition, modifications designed to eliminate aptamers that bind to the selection medium were made after 6 rounds:

- change from MPG-Streptactin beads to Sepharose–Streptactin beads (IBA GmbH);
- specific elution from the beads of 2′F-Py-ODNs/VCAM-1,2d complexes by desthiobiotine, before phenol/chloroform extraction.

Restriction fragment length polymorphism (RFLP) analysis, using a combination of RsaI, AluI, HaeIII, and HinPI endonucleases, was conducted to monitor the evolution of the sequence population, and revealed appearance of a discrete profile after 4 rounds, which evolved significantly between rounds 7 and 9 in response to the increased stringency (Fig. 2). Individual aptamers from the final pool (after 12 rounds) were monocloned in *Escherichia coli* using TopoTA cloning kit (Invitrogen). Twenty-five clones were randomly picked for DNA sequence analysis performed with Clustal X (Fig. 3),[22] which revealed the predominant selection of

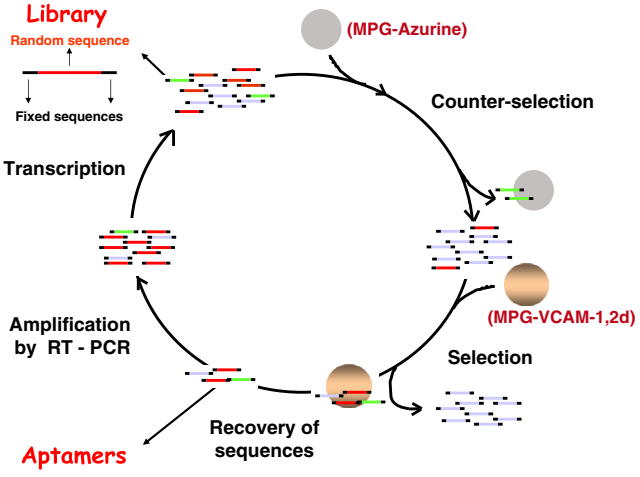

Figure 1. SELEX strategy: recombinant proteins for the couter-selection (Azurin) and for the selection (VCAM-1,2d) steps were immobilized on MPG beads to allow easy partionning of the bound and non-bound sequences pools.

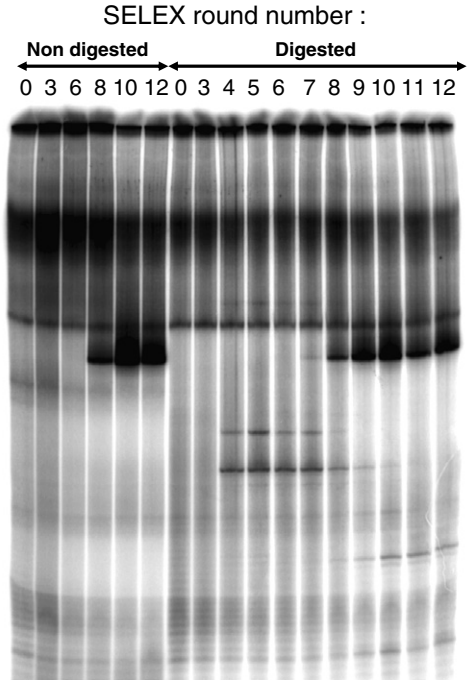

Figure 2. RFLP (Restriction fragment length polymorphism) profile during the selection process. SELEX round numbers are indicated above each lane, 0 stands for the starting library. Primer JCP40 was radiolabeled on its 5′-end by ^{32}P. Double-stranded DNAs from each round were digested with RsAI, AluI, HaeIII, and HinP1, while parallel samples were not. All were loaded onto a 15% denaturing gel. The preferential amplification of some sequences leads to the appearance of discrete bands.

Identifier	Sequence	Length
	10 20 30 40	
12.1	---TGCCGGAAGATGTT--AACAACCTCGACCTCACTCTTGGGC---	39
12.3	---TGCCGGAAGATGTT--AACAACCTCGACCTCACTCTTGGGC---	39
12.24	AAATCCCAGCCTCCAAC---GGGATTAAAACACTGCTGTTGG-----	39
12.28	AAATCCCAGCCTCCAAC---GGGATTAAAACACTGCTGTTG------	38
12.21	-----CCAGCCTCCTACCTTAAGTACTGTACCCTGCTGTTGGGGT--	40
12.5	-----AGGGAATCTTGGCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.18	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGACTCA--	39
12.11	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.31	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.15	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.14	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.13	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.7	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.12	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.17	-----AGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.25	------GGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	38
12.29	------GGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	38
12.26	-----TGGGAATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	39
12.19	------GGGGATCTTGCCTA-GGGAGGGAGTAGCGAAAGGGCTCA--	38
12.16	----GCCTCGGTCTTGTCTATGGGCAGGGATAGGGGTCGACCTC---	40
12.23	-----TCAGCAGTTCCGATAGGACCTTTGAGCGAACCCGAGATG---	39
12.4	----CTCACCAGCCGGCACGCCCACTGCTGTTGGACGGCGTGT----	39
12.10	----CGTACTGAGCCCGTTGAGTGCTTGTAGTACGTAGTCAAGT---	40
12.6	--TGATAAGCGGGGTGCTTAATACCCCTCGCGGTTCCTTAGC-----	40
12.27	-------CACCACTACTAGATCGGTGCATGCGGCACCATAGCTCCAC	40

Figure 3. Alignment of the 25 sequences cloned after 12 rounds of selection (primer regions are not represented).

one GA-rich family, accounting for 56% (14 over 25) of the sequences. The major aptamer among this family was aptamer 12.11, accounting for 32% (8 over 25) of the sequences. Interestingly, cloning after 7 rounds showed that this aptamer was already predominant (12.5%; 3 sequences over 24). Therefore, the increased stringency during the selection had favored its amplification.

Filter binding assay (FBA) was employed to further characterize the binding of individual 5′-^{32}P-labeled aptamers.[23] Assays were conducted on HAWP (45 μm) nitrocellulose filters (Millipore) pretreated with KOH 0.5 M, in the presence of salmon testes DNA (Sigma) as non-specific competitor to reduce baseline binding of oligonucleotides to the filter. Preliminary experiments confirmed that the main aptamer present in the final pool, 12.11, displayed the highest level of binding to VCAM-1,2d, while it did not bind to Azurine (expressed with *Strep*®-tag II), nor to an irrelevant protein like BSA (data not shown). Using either the initial pool or a scrambled sequence of the same base composition as

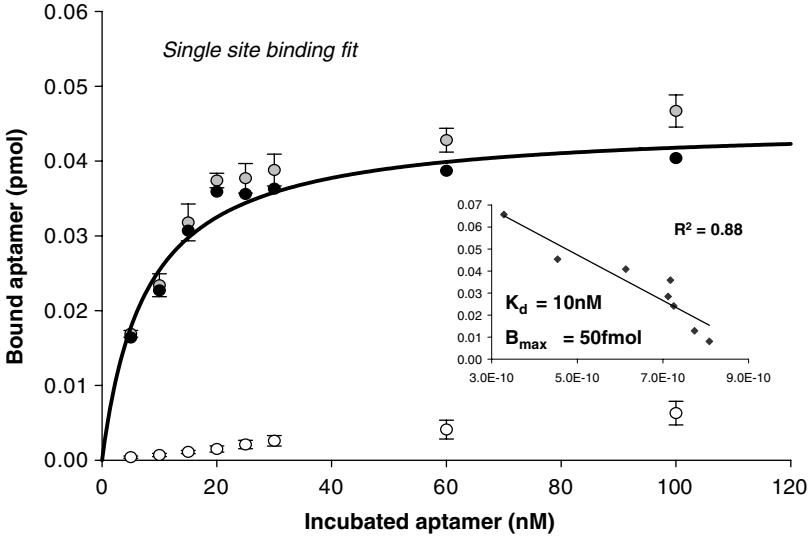

Figure 4. Representative FBA experiment with aptamer 12.11. Data are expressed as amount of bound aptamer (*Y* axis in pmol, mean ± SD, $n = 4$) for each concentration of aptamer (*X* axis in nM); gray: 12.11 binding, white: initial pool binding, black: specific binding calculated as "(12.11 binding) – (initial pool binding)" (gray minus white). Insert: Scatchard analysis.

that of 12.11 to estimate non-specific binding, Scatchard analysis of 12.11 binding to VCAM-1,2d yielded an affinity of 10 ± 5nM (Fig. 4).

Prediction of the secondary structure of aptamer 12.11 using MFOLD 3.1 software[24] indicated that stem–loop structures can be formed, as well as a guanine(G)-quartet, according to the sequence shown in Figure 3. We performed FBA experiments using potassium, sodium, or lithium-chloride buffers (150 mM): binding was increased in potassium buffer, compared with that in sodium and lithium buffers (respectively, by 31 ± 5% and 41 ± 9%).[25] This indicates that a G-quartet conformation may be responsible for the interaction with VCAM-1,2d.

Only a few aptamers have so far been selected against Ig or Ig-like targets.[26,27] Here we describe the selection of an high affinity aptamer against the N-terminal two-domain fragment of VCAM-1. This fragment corresponds to an accessible part of the protein expressed on the vascular endothelium, and is devoid of glycosylated residues in vivo.[28] Further work is now under way to develop in vivo applications of this versatile molecular tool in the imaging field, where 2′F-Py-ODNs have demonstrated high biodistribution potential.[29]

Acknowledgments

This work was supported by EU contracts QLG1-CT-2000-00562 (OLIM) and LSH-2004-503569 to the European Molecular Imaging Laboratory (EMIL) network, and by grant no. 3527 from the Association pour la Recherche sur le Cancer. F.C. is supported by a doctoral fellowship from the CEA. We wish to thank R. Souza for the gift of a $T7^{Y639F}$ RNA polymerase expressing plasmid.

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23. Prior to use, to disrupt intermolecular interactions, aptamer 12.11 was heated in RPMI medium at 85 °C for 5 min, cooled on ice for 5 min, and allowed to warm up at room temperature for 5 min. Increasing concentrations of aptamer 12.11 (5–100 nM, quadruplicates) were incubated with 20 pmol of VCAM-1,2d and 5 μg of salmon testes DNA at 37 °C in RPMI medium (total volume 50 μl), for 30 min. Filtration was followed by 12 consecutive washes by 500 μl RPMI. Radioactivity was quantified after exposition onto a PhosphorImager screen.
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