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Bioorganic & Medicinal Chemistry Letters 18 (2008) 1835–1839
Bioorganic & Medicinal Chemistry Letters

# Combinatorial selection of a single stranded DNA thioaptamer targeting TGF-β1 protein

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Received 20 August 2007; revised 8 February 2008; accepted 8 February 2008
Available online 13 February 2008

**Abstract**—A phosphorothioate single-stranded DNA aptamer (thioaptamer) targeting transforming growth factor-β1 (TGF-β1) was isolated by in-vitro combinatorial selection. The aptamer selection procedure was designed to modify the backbone of single-stranded DNA aptamers, where 5′ of both A and C are phosphorothioates, since this provides enhanced nuclease resistance as well as higher affinity than that of a phosphate counterpart. The thioaptamer selected from a combinatorial library ($5 \times 10^{14}$ sequences) binds to TGF-β1 protein with an affinity of 90 nM. In this report, sequence, predicted secondary structure, and binding affinity of the selected thioaptamer (T18_1_3) are presented.


Transforming growth factor-β1 (TGF-β1) is a 25 kDa homodimer composed of two 12.5 kDa subunits joined by a disulfide bond. The protein is known as a multifunctional cytokine acting both through autocrine and paracrine mechanisms. The protein suppresses the immune system and is particularly associated with immune disregulation by inhibiting the proliferation of normal T lymphocytes via down-regulation of interleukin-2-mediated proliferative signals.[1] TGF-β1 also mediates tumor-promoting effects, either through differential effects on tumor and stromal cells or through alteration in the TGF-β1 responsiveness of the tumor cells themselves.[2] Inhibition of the TGF-β signaling pathway has been proposed for cancer therapy.[3] Therefore, agents that target and antagonize TGF-β1 may serve as therapeutic or research tools by modulating the function of this protein.

Combinatorial selection of oligonucleotide aptamers has been employed to develop potential therapeutic agents since its introduction.[4,5] The principle of this technique is isolation and enrichment of specific nucleic acid sequences (aptamers) that bind to target molecules from a large random combinatorial library. This approach seems especially appropriate as inhibitor development because the protein is known to bind to heparin, a polyanionic polymer like DNA.[6] The selection in this study was designed to modify every 5′ of adenosine and cytosine of single-stranded DNA (ssDNA) aptamers to phosphorothioate, since phosphorothioate-substituted aptamers typically display an enhanced affinity for targeted molecules and increased stability in the biological milieu.[7,8] Several DNA[9,10] and RNA[11,12] phosphorothioate aptamers ('thioaptamers') have been isolated with these advantages.[13]

*Selection of ssDNA thioaptamers.* Among 42 independent sequences, three sequences (18_1_3, 18_1_11, and 18_2_2) were observed in multiple clones (Table 1). These more frequently selected sequences were chosen as thioaptamer candidates. Materials and methods are available in the online supplementary material.

*Sequence analysis selected thioaptamers.* As phosphorothioate modification has been known to enhance bind-

*Keywords*: Transforming growth factor-β1; Selection; Aptamer; Phosphorothioate; Thioaptamer; Chemiluminescence; Entropy.
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doi:10.1016/j.bmcl.2008.02.023

**Table 1.** Multiple sequence-alignment of thioaptamer candidates

| Name | Sequence |
|---|---|
| T18_1_3 (11) | --TGT**C**GT--TGT--GT**C**--**C**TGT**A**CCC**G**--**CC**TTG**A**CC**A**- |
| T18_1_2 (1) | TGT**C**T**C**G**A**--TG**C**T**A**G**AC**T-**C**T**A**TACCC**G**--**CCAA**------ |
| T18_1_1 (1) | --TGTGG**AC**-TG---GT**C**T-**A**TC**CA**TG**CA**--**CC**TGT**ACC**-- |
| T18_1_8 (1) | TGTGT-GT**A**-TG---GT**CC**-TTG**CA**T**C**G**A**TT**CCC**TG----- |
| T18_2_13 (1) | --TGT-GT**C**-T**A**----T**C**G-**C**TG**CACC**GTGT**CCA**T-**ACA**-- |
| T18_2_15 (1) | ---GT-GTG-**C**GTT-GT**C**---TGT--**CC**GTTT**CC**TGT**CCAC** |
| T18_2_10 (1) | TGTGG**C**GT--TG**A**T-**A**T**C**G**AC**TGT-------**CC**TTG-**CCAC** |
| T18_1_11 (2) | TTGGTTG**A**--TGTG**CA**T**C**G-**C**TGT-------T**CC**TGT**CCA**- |
| T18_1_4 (1) | TGGGT**C**G**C**--TG---**A**T**C**----G**CA**T**C**G**A**T**AC**T**C**T--**CCAC** |
| T18_1_24 (1) | **A**T**C**GT**C**G**AC**-TG----T**C**--**C**TGT**CAC**TGT-**CCA**T--**CCA**- |
| T18_2_2 (15) | --TGG**A**GG--TG**CC**TGG**A**---T**A**T**A**T**C**-G**A**--**C**T**C**G**ACCC**- |
| T18_1_10 (1) | -GTGT**CC**T--TGT**C**T**A**G**CC**T**C**G**A**T**A**-------**CAC**GA**CAC**- |
| T18_1_23 (1) | TGTGG**C**GT--TG----**AC**---TGT**ACAC**GT**C**G**A**T**ACACC**-- |
| T18_1_19 (1) | --TGG**C**TGG-T**CAC**TGT**A**--**C**T**C**T--**C**TG**C**-T**C**T**CCAC**--- |
| T18_1_13 (1) | --TGT**A**GTG-T**CC**-TGG**C**---T**A**T**CCAC**GT--**C**T**CCA**TT-- |
| T18_1_18 (1) | ----TGG**A**T-**C**G**C**TT**A**T**C**G**CC**T**C**G**A**T**C**-----**A**TTG**CCCA**- |
| T18_1_15 (1) | -TTGTTGT**AC**TGG**C**-**A**T**C**G**CC**T**C**G**AC**T**C**G---**C**TG------ |
| consensus | TGT G**A** TG GT**C** C**C**TGT**A** **C** G C**C** T G**ACC** |

42 sequences isolated independently at 18th round of selection were aligned using ClustalW algorithm. Number in the parenthesis indicates number of sequence. A and C, 5′ side of which are phosphorothioates, are in bold characters.

ing to proteins, the occurrence of the thioate linkage in the sequences was examined. We found that the thioate linkages occurred preferentially near the 3′ sides of the variable region of thioaptamer candidates. Localization of a thioate linkage at a particular position could be due to the property of the chemical nature of the modification that enhances protein-binding affinity.[9,12,13] This non-random occurrence of phosphorothioates in the thioaptamer candidates was further analyzed quantitatively. Clustering of phosphorothioates (A and C) in the variable region of the candidates was analyzed from the statistical thermodynamics point of view. Because thioate linkages occurred predominantly at 3′ side of the variable region, we examined the last 14 nucleotides of the region. The nucleotide composition entropy ($S_C$) can be calculated from its statistical weight ($W$)[14]

$$S_C = k \ln W \tag{1}$$

$$W = \frac{N!}{(n\mathrm{A} + n\mathrm{C})!(nG + nT)!} \tag{2}$$

where $k$ is Boltzmann constant ($= 1.380 \times 10^{-23}$ J K$^{-1}$), $N$ is the window size, fourteen in this case, and $nX$ is the number of nucleotide $X$ in the window. The corresponding free energy change ($\Delta G$) of clustering of phosphorothioates from a random sequence can be obtained from its entropy value as shown in Eq. 3:

$$\Delta G = T \times \Delta S_C \tag{3}$$

where $T$ is temperature in Kelvin and $\Delta S_C$ is difference of $S_C$ between a random sequence and a thioaptamer sequence. The results of these calculations are shown in Table 2. According to the calculation T18_1_3 has the lowest $S_C$ value. According to the secondary structure prediction, the variable region of T18_1_3 does not contain secondary structure elements (Fig. 1).

*Binding assay.* To identify the best thioaptamer, target-binding affinities of the thioaptamer candidates were examined by chemiluminescence (CL) electrophoretic mobility shift assay (EMSA) as CL IDV (integral density value) was identified to be linearly proportional to the sample quantity,[15] which has been applied to quantitative binding studies.[6,16,17] First, binding of the initial thioaptamer library to the protein was assessed using CL EMSA (Fig. 2). As the concentration of the protein in a binding mixture increased, the DNA bands (labeled as T and B in Fig. 2) of the initial thioaptamer library in the EMSA image decreased and shifted bands were observed near the well of the gel. Because the CL intensity of the shifted band was much less than that of free DNA, the decrease of the main band rather than the

**Table 2.** Statistical thermodynamic analysis of selected thioaptamers and a random sequence

| Sequence | $W$ | $S_C$ (JK$^{-1}$) | $\Delta G$ (kJ mol$^{-1}$) |
|---|---|---|---|
| T18_1_3 | 1,001 | $0.95 \times 10^{-22}$ | 3.05 |
| T18_1_11 | 3,003 | $1.11 \times 10^{-22}$ | 0.33 |
| T18_2_2 | 2,002 | $1.05 \times 10^{-22}$ | 1.33 |
| Random | 3,432 | $1.12 \times 10^{-22}$ | 0 |

$\Delta G$ was obtained for 298 Kelvin.