Supplementary data

Combinatorial selection of a single stranded DNA thioaptamer targeting TGF-beta1 protein

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Materials and methods

Library and PCR: A random combinatorial single-stranded DNA (ssDNA) library of normal phosphoryl backbone oligonucleotides (74-mer) was synthesized (Midland Certified Reagents, Midland, TX): 5′-CAGTCCGGATGCTCTAGAGTGAC$[N]_{30}$CGAA TCTCGTGAAGCCGAGCG-3′ where N is a randomized nucleotide with equal proportion of A, G, C, T. The library was replicated using Klenow Fragment DNA polymerase and subsequently amplified using *Taq* polymerase (Amplitaq, Perkin-Elmer). The library with phosphorothioate backbone substituted at A and C positions was then synthesized by PCR amplification of the template using *Taq* polymerase and a mixture of dATP (αS) (Amersham Biosciences), dTTP, dGTP and dCTP (αS) (Amersham Biosciences) as substrates. The PCR condition for amplification of the starting random library (5×10^{14} sequences) includes 200 μM each of dATP (αS), dTTP, dGTP, and dCTP (αS), 4 mM $MgCl_2$, 740 nM 74-mer random template, 50 units of *Taq* polymerase, and 2.4 μM each primer in a total volume of 1 ml. PCR was performed to amplify the

selected DNA with biotin-conjugated 5′ primer (biotin-biotin-biotin-5′-CAGTCCGGAT GCTCTAGAGTGAC-3′) and 3′ primer (5′-CGCTCGGCTTCACGAGATTCG-3′) (Midland) under the following conditions: 94 °C for 5 min; 40 cycles at 94 °C for 1 min, 65 °C for 2 min, and 72 °C for 3 min; the final extension was at 72 °C for 10 min. This polymerase acts stereospecifically to incorporate the S_p-diastereomers of dNTP (αS) and is believed to produce the R_p stereoisomer as is found for other polymerases.[1]

Selection of ssDNA thioaptamers: The 74-nucleotide PCR product (~ 1 nmol) was applied to 400 µl of a Magnetic Porous Glass (MPG) Streptavidin (CPG Inc.) (10 mg/ml, 4-6 $\times 10^7$ particles/ml) bead matrix suspended in Binding/Wash buffer (2.0 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The mixture was gently rotate at room temperature for 1 h. After equilibration of binding of the biotinylated double-stranded DNA (dsDNA) to streptavidin beads, unbound dsDNA was removed with 900 µl of Binding/Wash buffer (2 times), and the matrix-bound dsDNA was denatured in 150 µl of Melting solution (0.1 M NaOH) for 10 min at room temperature and washed one time with 150 µl of Melting solution. As these conditions were not harsh enough to break the biotin-streptavidin interaction, this denaturation step released only nonbiotinylated ssDNA strand from the bead complex.[2,3] To remove NaOH in the sample and to collect the released nonbiotinylated ssDNA, the supernatant of the sample was filtered with a Microcon YM-10 filter (Millipore) and washed with PBS buffer several times, yielding 0.1-0.3 nmol of ssDNA. The purity of the ssDNA library was confirmed by amplification of the correctly sized product with the forward primer but not the reverse primer after 5 cycles of PCR. Generally, 0.1-0.3 nmol of the enriched ssDNA was used for the

following round of combinatorial selection. This enriched ssDNA was denatured and renatured by incubating it at 95 °C for 10 min and slowly cooling to room temperature with addition of $MgCl_2$ at final concentration to be 1 mM. This renatured ssDNA was incubated with TGF-β1 protein (Peprotech) in PBS/ 1mM $MgCl_2$ usually at room temperature for 30 min and filtered through MF-Millipore nitrocellulose membrane filters (0.45 μm pore size, filter diameter 13 mm) previously presoaked in PBS. Under these conditions, the DNA/protein complexes were retained on the filter. The filter was then washed with 10 ml of PBS to remove the majority of the DNA, which only weakly bound to the protein. To elute the protein bound DNA the filter was incubated in elution solution containing 8 M urea at 70 °C for 10 min. To remove urea in the solution and to collect the protein-bound DNA, the supernatant of the sample was filtered with a Microcon YM-10 filter (Millipore) and washed with PBS buffer several times. The DNA retained on the filter was put to PCR to generate a DNA library for the next round selection. In 18th round PCR, 5% DMSO and 1 M betaine were added into the PCR mixture to prevent secondary structure formation.[4] To increase aptamer selectivity, later selection rounds included increasing concentrations of NaCl up to 210 mM in the binding buffer. The detailed condition for selection and PCR amplification is shown in Supplementary Table 1. DNA from the 5, 9, 12, and 18 rounds of selection, as well as the initial library, were cloned using the TOPO cloning kit (Invitrogen) and sequenced.

Analysis of thioaptamer sequences: Multiple sequence alignment of thioaptamer candidates was conducted using ClustalW 1.8 (http://searchlauncher.bmc.tmc.edu) with

20 for both the gap opening and extension penalties. Prediction of ssDNA secondary structure was conducted using *mfold*.[5]

DNA biotinylation and EMSA: ssDNA was biotin-labeled at the 3′ end using Biotin 3′ End DNA Labeling Kit (Pierce) following the manufacturer's protocol with few modifications. Before the labeling, ssDNA was heated at 95 °C for 10 min and quickly cooled by placing on ice. The labeling reaction was conducted at 37 °C for 2 h. After biotinylation, ssDNA was denatured and renatured by incubating it at 95 °C for 10 min and slowly cooling to room temperature with addition of $MgCl_2$ as final concentration to be 1 mM. The binding affinity of the TGF-β1 proteins to ssDNA was analyzed using the electrophoretic mobility shift assay (EMSA). 4 nM of biotinylated ssDNA was incubated with a variable quantity of TGF-β1 in PBS/1mM $MgCl_2$ at room temperature for 30 min. Subsequent EMSA was done as described previously.[6] A cooled charge-coupled device (CCD) camera (Fluor Chem 8800 Imaging system) purchased from Alpha Innotech (San Leandro, CA) was used for image capture and measurement of chemiluminescence (CL) integrated density value (IDV).[7] Binding of the DNA to the protein was assessed by measuring the decrease of CL IDV of the unbound DNA as the protein was added to the reaction mixture.[6] Curve fittings of IDV data were conducted using SigmaPlot2001 (SPSS Inc., Chicago, IL).

DNA-DNA titration: Biotin-labeled thioaptamer 18_1_3 of a constant quantity was mixed with a variable quantity of unlabeled 18_1_3 in 80 μl of the binding buffer. The mixture was heated at 95 °C for 10 min and slowly cooled to room temperature; 20 μl of the mixture was analyzed by CL EMSA.

Supplementary Table 1

Selection and amplification conditions

Round	[DNA] (μM)	[P] (nM)	[Na] (mM)	BRV (μl)	PCR
1	1.42	320	0	260	40 (1.0)
2	0.91	310	0	160	40 (1.0)
3	1.56	200	0	170	40 (1.0)
4	0.15	110	0	120	40 (1.0)
5	0.74	50	0	110	40 (1.0)
6	0.26	30	0	200	40 (1.0)
7	0.12	30	12	200	40 (1.0)
8	0.34	30	24	200	40 (1.0)
9	0.43	30	37	200	40 (1.0)
10	0.79	30	52	200	40 (1.0)
11	0.41	30	67	200	40 (1.0)
12	0.54	30	84	200	40 (0.8)
13	0.23	30	101	400	35 (0.8)
14	0.19	30	120	400	30 (0.8)
15	0.29	30	140	400	25 (0.8)
16	0.45	30	162	400	20 (0.6)
17	0.21	30	185	400	18 (0.6)
18 (1)	0.16	30	210	400	16 (0.5)
18 (2)	0.18	30	210	400	16 (0.5)

[P] is the concentration of TGF-β1 in the binding reaction. [Na] is the concentration of NaCl added to the binding mixture in extra. BRV is binding reaction volume. The number of PCR cycle is shown in the PCR column and the number in the parenthesis is PCR volume (ml). Betaine and DMSO were added to the PCR mixture of round 18 as noted 18(2).

References

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