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Aptamer selection based on inhibitory activity using an evolution-mimicking algorithm

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Abstract

In order to efficiently select aptamers that bind to and inhibit proteins, we developed a method that involves screening DNA aptamers based on their inhibitory activities using an evolution-mimicking algorithm after the pre-selection by SELEX. The value of this method was demonstrated by the identification of an inhibitor of *Taq* DNA polymerase in a unique single-stranded DNA library, which was expected to form a G-quartet structure. This method consists of selection via an inhibition assay, sequence shuffling, and mutation *in silico*. After six rounds of selection, the inhibitory activities of the aptamers had evolved significantly. This demonstrates the utility of this strategy for screening aptamers based on their inhibitory actions.

Keywords: Evolution-mimicking algorithm; G-quartet library; Aptamer; SELEX; Inhibitor

Aptamers, which are known to bind to proteins with high affinity and specificity, are currently the subject of many studies focused on their abilities to inhibit enzymes. Some aptamers have potent inhibitory activities (e.g., K_i values of the order of nanomolar range) [1–3]. However, the method used to select aptamers, systematic evolution of ligands by exponential enrichment (SELEX) [4,5], does not directly lead to aptamers that function as enzyme inhibitors, since this method is based on affinity selection, which does not prevent the selection of aptamers that bind to functionally unimportant regions of the protein.

Ideally, for the discovery of therapeutic agents, selection should be based on the inhibitory activities of aptamers. Since it is not possible to measure the inhibitory activities of all the oligonucleotides present in a diverse library, we focused on the use of genetic algorithms (GAs) [6] for the selection of aptamers based on their inhibitory activities. We previously reported the identification of a peptide inhibitor of trypsin and 15-mer peptides that formed an α-helix [7,8]. To further improve the screen for inhibitors, various GAs were combined with the concept of exon shuffling, which resulted in the development of a novel method for efficient searching in the sequence spaces by shuffling of functional blocks among multiple sequences. Using this method, which is called the evolution-mimicking algorithm (EMA), DNA aptamers that inhibit thrombin have been selected [9].

However, in these screenings, the GAs and EMA were applied to relatively small libraries that contained $\leqslant 10^5$ sequences. In order to expand the potential of EMA-based screening, a screening method that selects aptamers from a library that contained $\geqslant 10^{10}$ sequences was developed in the present study. This method consists of a two-stage screen that combines conventional affinity selection by SELEX and selection using EMA based on the inhibitory activity of each aptamer. Although previous studies have demonstrated the efficiency of EMA in the screening of inhibitors [9], it was difficult to identify the optimal inhibitor sequence through synthesis and the evaluation of a

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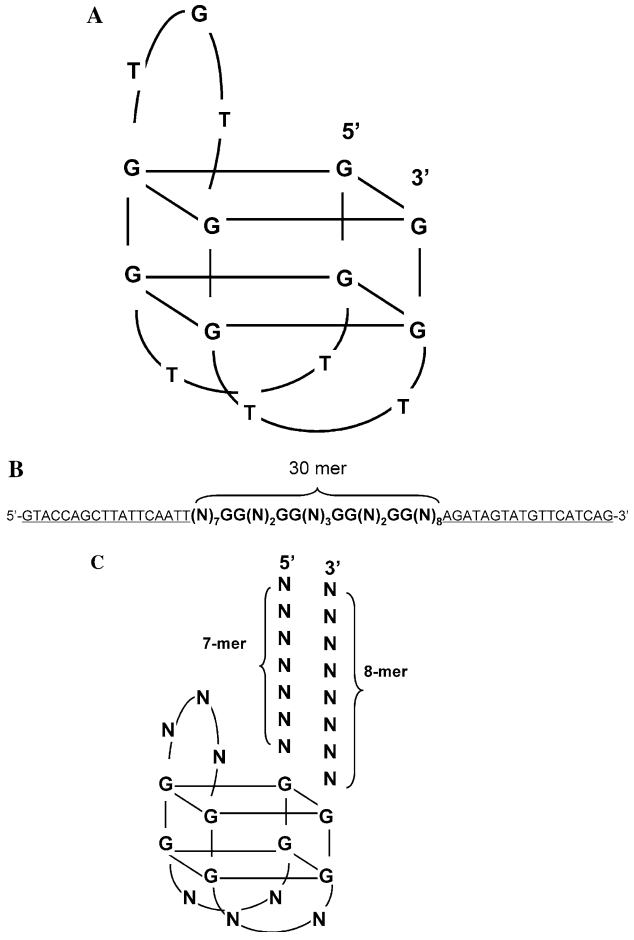

Fig. 1. Sequence and structure of the G-library. (A) Structure of TBA. (B) Whole sequence of the G-library. Underlined regions are the primer binding sites. (C) Putative structure of the G-library.

large library that contained $\geqslant 10^{10}$ sequences. The number of sequences assayed by EMA in each generation (usually 10–20) is low relative to the number of sequences in the library. However, the removal of vast numbers of unbound species and the selection of candidates to synthesize and evaluate from the large library using SELEX can improve the applicability of EMA to the screening of large libraries.

In order to demonstrate the potential of this strategy, aptamers that inhibit *Taq* DNA polymerase (*Taq* pol) were screened from a unique single-stranded DNA library (G-library), which was designed to form a G-quartet structure (Fig. 1). Since *Taq* DNA polymerase interacts with nucleic acids *in vivo* and it is known that the DNA aptamer, Trnc.A-30, was obtained using conventional SELEX and strongly inhibited this enzyme [1,10], this enzyme was chosen as a model to investigate the effectiveness of our method.

Materials and methods

Materials. Oligonucleotides and those labeled with FITC or 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX) were synthesized by standard solid-phase chemical synthesis (Invitrogen). The PCR reagents and *Taq* pol were purchased from Takara.

Pre-selection using SELEX. SELEX was performed as described previously [1], with the following modifications. The synthetic random sequence DNA pool (1.5 nmol) was suspended in the selection buffer (50 mM KCl, 2.5 mM $MgCl_2$, and 10 mM Tris–HCl at pH 8.3) and incubated with *Taq* pol for 15 min. The *Taq*-binding library was obtained by filtering with a nitrocellulose membrane (Millipore). The isolated library was amplified by PCR with the primers 5′-GTACCAGCTTATTC AATT-3′ and 5′-BCTGATGAACATACTATCT-3; where B stands for biotin [11]. The ssDNA library was prepared with avidin-immobilized gel beads. The fifth- and sixth-round libraries were subcloned into the pGEM-vector (Promega) and the clones were sequenced.

Gel mobility shift assay. To evaluate the affinities of the oligonucleotides, 50 nM of 5′-HEX-labeled oligonucleotides was incubated with 50 nM *Taq* pol at 25 °C for 15 min. The mixtures were loaded on 2% agarose gels in 0.5× TBE buffer and subjected to electrophoresis at 50 V. The intensity of the retarded band for DNA bound to *Taq* pol was quantified with the Typhoon 8600 fluorescence scanner (Amersham Biosciences) and ImageQuant software (Molecular Dynamics). The K_d (equilibrium dissociation constant) values were estimated using the equation described previously [12].

Measurements of inhibitory activity. These measurements were carried out using the hairpin extension assay described previously [1], with the following modifications. The 20 μl reaction mixture contained 6.25 nM *Taq* pol, aptamers (at varying concentrations), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM of each dNTP. The hairpin DNA substrate was 5′-labeled with FITC and added to a final concentration of 31 nM. The extension products were resolved in 15% (w/v) polyacrylamide gels that contained 7 M urea. The ratios of elongated and non-elongated products in the presence and absence of the aptamers were quantified with Typhoon 8600 system and ImageQuant software.

EMA. EMA-based screening after SELEX was carried out as described previously [9], with the following modifications (Fig. 2). A set of ten 30-mer DNA sequences from the G-library was randomly generated by a computer and synthesized. The inhibitory activity of each aptamer for *Taq* pol was measured as described above and ranked accordingly. Parent aptamers and their selection ratios were manually determined based on their sequence homologies and activities. Subsequently, the sequences of five sequence blocks were (Fig. 2B) shuffled among the selected aptamers, respectively, which was followed by the generation of 10 sequences. Finally, point mutations were introduced into these 10 sequences at a certain constant frequency. The oligonucleotide 6G21 in the first generation carried a mutation in the G-quartet region. This mutation was then corrected and used to generate the second generation.

Surface plasmon resonance. For surface plasmon resonance (SPR), aptamer 6–10 was 5′-biotinylated and immobilized on a streptavidin-containing surface (BIACORE) at the level of ~1000 response units. The running buffer, which was also the selection buffer, contained 5 μM EDTA, 50 μM DTT, 0.025% Tween 20, and 2.5% glycerol. The K_d values were determined with the Prism software (GraphPad Software).

Results

Library design and removal of unbound species using SELEX

The G-library, which was designed to form a G-quartet structure (Fig. 1), was used in this study, although the DNA aptamers predicted to form a stem-loop structure were obtained from a completely random pool [1,10]. Various aptamers are known to form the G-quartet structure [2,3,13–16]. Studies on the thrombin-binding aptamer (Fig. 1A) and integrase-binding aptamer have suggested

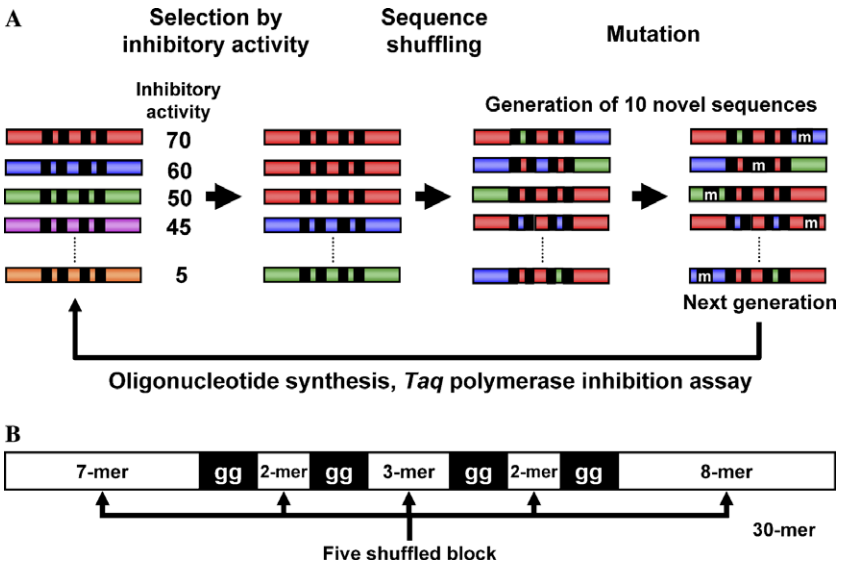

Fig. 2. Scheme of the screening using the EMA. (A) Procedure of the EMA. (B) Position of functional regions and structural regions in an aptamer.

that guanine quartets form the overall structure and that the loop regions play a key role in molecular function, similar to the scaffold and loops, respectively, of an antibody [2,3,17]. Therefore, this structure was chosen as the scaffold for the aptamers for *Taq* pol; randomization of the loop regions and the addition of seven and eight random nucleotides to the 5′- and 3′-ends, respectively, could be used to modulate the affinity and inhibitory activity towards *Taq* pol. The G-library was designed to contain a contiguous random 22-nucleotide region and four blocks of two guanines in the center (Fig. 1B), based on the thrombin-binding aptamer, which is referred to as TBA (Fig. 1A) [17]. Since the G-quartet structure is known to be relatively stable in the presence of metal ions [3], some of the oligonucleotides in this library were expected to form TBA-like G-quartet structures in selection buffer that contained 50 mM K^+.

In order to eliminate non-candidate oligonucleotides from the selection process, SELEX was used initially to reduce the number of sequences submitted to the EMA. The number of binding species for *Taq* pol was gradually increased by SELEX, and the recovery of the input library was >20% by the sixth round (data not shown). Therefore, it appears that many of the non-binding species had been removed at this stage. Each clone in the selected libraries at the fifth and sixth round was sequenced, and the affinity and inhibitory activity of each oligonucleotide were assayed by the gel mobility shift assay and hairpin extension assay [1]. Since the sequences of the SELEX-selected oligonucleotides showed no obvious homologies among the 29 clones (Table 1), the selected library appeared to retain sequence complexity. However, some of these aptamers bound to and inhibited *Taq* pol (Fig. 3). The K_d values of the aptamers with the highest (62%) and lowest (28%) affinities were estimated at approximately 13 and 80 nM, respectively. The IC_{50} values (the concentration of inhibitor required to produce 50% of the product in the assay) of the aptamers with the highest (69%) and lowest (below the level of detection) inhibitory activities were estimated as <1 and >10 μM, respectively (data not shown). Thus, the inhibitory activities of the aptamers were significantly independent of their affinities for *Taq* pol. Not

Table 1
Sequences of oligonucleotides in the fifth and sixth round library

Clone number	Sequence
5G1	CCAGACG ggTAggCGTggGGgg TTACTTGG
5G3	GGATAAG ggAAggGA ggAAgg CAGTGTTT
5G4	GTATGTG ggGGggATTggGTgg CCGTTATC
5G5	CAAGGAC ggAGggCGAggGTgg GTTACTGA
5G8	AGTAACG ggAAggTAGggTTgg TTGTGGAT
5G9	CGAAAGG ggGGggTGTggTCgg AAAGTGGC
5G11	CGCGTGG ggAGggAGAggATgg GTCCGTT
5G12	ACAGGCG ggTGggAGAggTCga GAGGGGGT
5G14	GGGGACT ggAAAggTAggTTgg GACATGAT
5G15	GTGTCCG ggTAggTAAggGTgg CTATAGCA
5G16	GCTATTA ggTAggAGAggCGgg ATTCACTC
5G18	CAGTAGT ggATggGA ggTGgg CTTACGCG
5G19	TCGGAGG ggATggGTGggTAgg TCCGTGTT
5G21	ACAAAGC ggTAggTAGggTCgg TATTTCTG
5G22	ACGGTCT ggGGggTGTggTAgg CGCCGTCT
6G1	GCCCGAT ggTAggACCggATgg GGTGTTT
6G3	CCCGCAC gaCGgtGAGgtTGgg TGTATGT
6G4	CGGTGGT ggTGggTTAggACgg TATTGGGC
6G5	ACATGCA ggGTggGAAggCAgg TTTCTCGT
6G6	ACATGAT ggTCggATTggTTgg CGCCCTAG
6G7	GGGTCAG ggAAggAAGggAGgg TAGCCGTC
6G8	TCGGGTG ggTAggAGGggTTgg AGGCGCAT
6G9	CGGCAAT ggAAggTTAggGTgg TTTGCATG
6G10	GGGAAAG ggGAggACGggACgg ATGGAGCT
6G11	ACAATAG ggTGggGGCggGCgg TGTGCTTG
6G13	ACACTAC ggTGggTATggAAgg TTTAACGT
6G14	CGGGGGG ggATggCAGggGTgg CGTCATTG
6G20	ACATTTC ggGTggAGTggGGgg AGTTCAGT
6G21	GGCAAAT ggCGgtTATggGCgg TTATGTTA

Oligonucleotides whose name start with "5" and "6" are oligonucleotides in the fifth and sixth round library, respectively. Small letters depict the bases corresponding to guanines that form G-quartet structure.

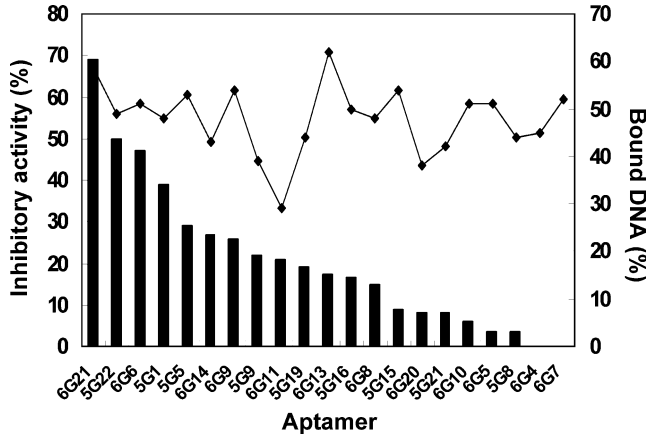

Fig. 3. Relationship between affinities and inhibitory activities of aptamers. A bar graph and a line graph indicate inhibitory activities of aptamers and ratio of DNA bound to *Taq* pol, respectively. The inhibition assay was performed in 2.5 nM *Taq* pol and 1250 nM aptamer.

all of the aptamers with higher affinity show stronger inhibitory activity and vice versa, which indicates that aptamer binding ability did not always correlate with aptamer inhibitory activity. Thus, it was decided to terminate the SELEX selection after the sixth round, and the EMA-based screening was initiated in the form of synthesis and evaluation in this *Taq*-binding library of the inhibitory activities of each aptamer using four aptamers with higher inhibitory activities (Fig. 3).

Screening based on inhibitory activity using EMA

The inhibitory activities of the aptamers are shown in Fig. 4. Although the screening cycle was repeated six times, the inhibitory activities of the aptamers from the six generation are excluded from this figure, since the conditions used for the measurement of inhibitory activity in the sixth generation were slightly different (lower aptamer concentration of 500 nM) from those used in the other generations. As expected, the inhibitory activities of aptamers increased with repetition of the EMA-based screening.

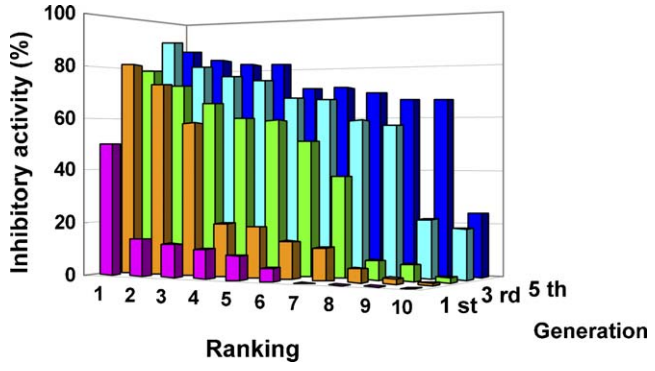

Fig. 4. Inhibitory activity of each aptamer in each generation (1st–5th). The inhibition assay was performed in 6.25 nM *Taq* pol (higher than the assay for Fig. 3 by 2.5-fold) and 1250 nM aptamer.

The average and maximum activities of the aptamers rose to more than 60% and 30%, respectively. Although several ssDNA molecules may exist that inhibit *Taq* pol, the inhibitory activities of the aptamers increased with the use of EMA. Therefore, these data demonstrate that screening based on inhibitory activity (as opposed to affinity) by EMA is efficient and effective for the screening of aptamers.

Aptamers with potent inhibitory activities from the second to fifth generations were assayed under the same conditions as the aptamers from the sixth generation (Table 2). Among these aptamers, the consensus sequence CGCCVTMK was identified in the 3′-region (depicted by IUB codes) and a conserved CG motif was noted in the first loop of the G-quartet. In addition, these aptamers carried the conserved TKT and TA motifs in the second and third loop regions of the G-quartet, respectively. The sequences in the 5′-region consisted of ACTTGAT and CAAGACG.

The IC_{50} value of the most potent aptamer identified by the EMA, aptamer 6–10 (Table 2), was also determined. The IC_{50} values of aptamers 6–10 and Trnc.A-30, which was obtained by conventional SELEX and shown to inhibit *Taq* pol activity [1,10], were 117 and 67 nM, respectively, under the conditions used in this experiment. Aptamer 6–10 showed potent inhibition, with an IC_{50} value was comparable (less than a 2-fold difference) to that of Trnc.A-30 obtained by SELEX.

The affinity of aptamer 6–10 for *Taq* pol was measured by SPR, and the K_d value was calculated as 5.7 nM. It is possible that the aptamers obtained using the EMA also bind to the substrate DNA used for the hairpin extension assay. However, the assay was performed in the absence of the substrate, and aptamer 6–10 inhibited *Taq* pol in an assay using a different substrate (data not shown), which demonstrates that aptamer 6–10 binds with high affinity to *Taq* pol.

Discussion

Our selection method using EMA is suitable for the screening of aptamers as inhibitors, since it selects aptamers based on their inhibitory activities. Affinity selection methods, such as SELEX, efficiently select artificial ligands for various target molecules by simultaneously assaying the individual molecules in a library for affinity. However, aptamers that show inhibitory activities and that may have value as therapeutic agents are not always selected by SELEX. Affinity selection alone can select aptamers that bind to functionally unimportant regions of a protein that have little inhibitory activity. Indeed, the aptamers selected against human neutrophil elastase and farnesyltransferase bound to the targets with K_d values in the nanomolar range but did not inhibit the activities of these enzymes [13,18]. Similarly, although the 29-mer thrombin aptamer had 20- to 50-fold higher affinity than the 15-mer thrombin aptamer, it had less than half the inhibitory activity [19]. Therefore, in the case of aptamers that are expected to

Table 2
Total rank of the inhibitory activities of the aptamers

Rank	Aptamer	Inhibitory activity (%)	Sequence
1	6–10	83 ± 1	CAAGACG ggCGggTGTggTAgg CGCCCGTG
2	4–1	82 ± 1	ACTTGAT ggCGggTGTggTAgg CGCCATCT
3	6–8	80 ± 2	ACTTGAT ggCGggTTTggTAgg CGCCGTCG
4	6–5	77 ± 1	ACTGGAT ggCGggTTTggTAgg CGCCATCT
5	6–4	76 ± 1	ACATGAT ggCGggTGTggTAgg CGTCGTCT
6	2–4	75 ± 2	ACATGAT ggCGggTTTggTAgg CGCCGTCT
7	5–8	72 ± 1	CAAGACG ggCGggTTTggTAgg CGCCCGAG
8	6–1	71 ± 1	CAAGACG ggCGggTTTggGAgg CGCCCGAG
9	5–7	64 ± 0	ACTTGAT ggCGggTTTggTAgg CGCCCTCT
10	3–3	63 ± 2	CAAGACG ggCGggTTTggTAgg CGCCGTCT
11	4–8	61 ± 3	ACTTGAT ggCGggTTTggTAgg CGCCCCAG
12	5–4	60 ± 1	ACTTGAT ggTGggTTTggTAgg CGCCGTCT
13	5–10	60 ± 0	ACAGACG ggCGggTTTggTAgg CGCCGACT
14	4–3	58 ± 3	CCTGACG ggCGggTTTggTAgg CGCCGTCT
15	4–7	58 ± 3	ACAGACG ggCGggTTCggTAgg CGCCCTAG

The inhibition assay was performed in 6.25 nM *Taq* pol and 500 nM aptamer. Each aptamer was designated *X*–*Y*, where *X* is the number of the generation and *Y* is the clone number.

act as inhibitors, selection should be based on aptamer inhibitory activity; our selection method using EMA is useful in this regard.

In order to demonstrate the efficacy of the screening using EMA, we screened for DNA aptamers that inhibit *Taq* pol using the EMA. Excellent aptamers with potent inhibitory activities were identified by the EMA that were not obtained by the conventional SELEX method. The inhibitory activity of Trnc.A-30, which was selected using SELEX, was almost the same as that of aptamer 6–10. However, it should be pointed out that the starting library for Trnc.A-30 theoretically contained 10^{18} sequences, while that of aptamer 6–10 contained only 10^{13}. In general, it is believed that the more diverse the library the better the selected aptamer. Therefore, it is not surprising to discover that the SELEX-derived aptamers from a library that contained 10^{18} sequences has significantly better activity than those selected from a smaller library with a constrained structure. Nevertheless, we have successfully selected aptamers with potent inhibitory activities from a smaller library using EMA. This indicates that the EMA-based selection method selects aptamers with inhibitory activities that an affinity selection method, such as SELEX, cannot provide. Therefore, if selection using EMA was performed on a larger library, such as the starting library for Trnc.A-30, superior aptamers might be obtained.

We did not investigate whether the aptamers obtained by EMA actually formed the G-quartet structure, as our aim of identifying good inhibitory aptamers with EMA was achieved. Some aptamers may form the G-quartet structure, while other aptamers may not. The crucial point is that the EMA-based method identifies DNA aptamers with inhibitory activities. Nonetheless, it is important to investigate the G-quartet structure formation for the design of superior inhibitory aptamers and its investigation might be reported elsewhere.

In principle, EMA is capable of driving the evolution of any function as long as the desired function can be assayed and ranked for example, using the parameters of specificity of inhibition and efficacy *in vivo*. The *in vivo* and *in vitro* inhibitory activities of aptamers do not always correlate, and modifications to the original aptamers are often necessary [20].

Acknowledgments

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References

[1] C. Dang, S.D. Jayasena, Oligonucleotide inhibitors of Taq DNA polymerase facilitate detection of low copy number targets by PCR, J. Mol. Biol. 264 (1996) 268–278.
[2] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, Nature 355 (1992) 564–566.
[3] N. Jing, C. Marchand, J. Liu, R. Mitra, M.E. Hogan, Y. Pommier, Mechanism of inhibition of HIV-1 integrase by G-tetrad-forming oligonucleotides in vitro, J. Biol. Chem. 275 21460–21467.
[4] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 249 (1990) 505–510.
[5] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822.
[6] D.E. Goldberg, Genetic Algorithms in Search Optimization and Machine Learning, Addison-Wesley, Massachusetts, 1989.
[7] Y. Yokobayashi, K. Ikebukuro, S. McNiven, I. Karube, Direct evolution of trypsin inhibiting peptides using a genetic algorithm, J. Chem. Soc., Perkin. Trans. I 20 (1996) 2435–2438.
[8] W. Zhang, M.G. Loughran, S. Kanna, K. Yano, K. Ikebukuro, Y. Yokobayashi, R. Kuroda, I. Karube, Exploration of structural features of monomeric helical peptides designed with a genetic algorithm, Proteins 53 (2003) 193–200.
[9] K. Ikebukuro, Y. Okumura, K. Sumikura, I. Karube, A novel method of screening thrombin-inhibiting DNA aptamers using an evolution-mimicking algorithm, Nucleic Acids Res. 33 (2005) e108.

[10] Y. Lin, S.D. Jayasena, Inhibition of multiple thermostable DNA polymerases by a heterodimeric aptamer, J. Mol. Biol. 271 (1996) 100–111.
[11] T. Kato, K. Yano, K. Ikebukuro, I. Karube, Interaction of three-way DNA junctions with steroids, Nucleic Acids Res. 28 (2000) 1963–1968.
[12] T. Fitzwater, B. Polisky, A SELEX primer, Methods Enzymol. 267 (1996) 275–301.
[13] R.H. Shafer, I. Smirnov, Biological aspects of DNA/RNA quadruplexes, Biopolymers 56 (2000) 209–227.
[14] Y. Lin, A. Padmapriya, K.M. Morden, S.D. Jayasena, Peptide conjugation to an in vitro-selected DNA ligand improves enzyme inhibition, Proc. Natl. Acad. Sci. USA 92 (1995) 11044–11048.
[15] J.C. Darnell, K.B. Jensen, P. Jin, V. Brown, S.T. Warren, R.B. Darnell, Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function, Cell 107 (2001) 489–499.
[16] T. Mori, A. Oguro, T. Ohtsu, Y. Nakamura, RNA aptamers selected against the receptor activator of NF-kappaB acquire general affinity to proteins of the tumor necrosis factor receptor family, Nucleic Acids Res. 32 (2004) 6120–6128.
[17] K. Padmanabhan, K.P. Padmanabhan, J.D. Ferrara, J.E. Sadler, A. Tulinsky, The structure of alpha-thrombin inhibited by a 15-mer single-stranded DNA aptamer, J. Biol. Chem. 268 (1993) 17651–17654.
[18] M. Berezovski, A. Drabovich, S.M. Krylova, M. Musheev, V. Okhonin, A. Petrov, S.N. Krylov, Nonequilibrium capillary electrophoresis of equilibrium mixtures: a universal tool for development of aptamers, J. Am. Chem. Soc. 127 (2005) 3165–3171.
[19] D.M. Tasset, M.F. Kubik, W. Steiner, Oligonucleotide inhibitors of human thrombin that bind distinct epitopes, J. Mol. Biol. 272 (1997) 688–698.
[20] L.A. Cassiday, L.J. Maher 3rd, Yeast genetic selections to optimize RNA decoys for transcription factor NF-kappa B, Proc. Natl. Acad. Sci. USA. 100 (2003) 3930–3935.