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A novel lipopolysaccharide-antagonizing aptamer protects mice against endotoxemia

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ABSTRACT

A growing number of researchers have recognized the importance of using lipopolysaccharide (LPS) as target for the prevention and treatment of sepsis. However, no drugs targeting LPS have been applied clinically. In this study, LPS-inhibiting aptamers were screened by Systematic Evolution of Ligands by Exponential Enrichment (SELEX), and their therapeutic effects for experimental sepsis were observed. After 12 rounds of screening, 46 sequences were obtained. Primary structure analysis indicated that they had identical sequences, partly conserved sequences, or non-conserved sequences. Secondary structure analysis showed these sequences usually contained hairpin or stem–loop structures. Aptamer 19 significantly decreased NF-κB activation of monocytes challenged by LPS and reduced the IL-1 and TNF-α concentration in the media of LPS-challenged monocytes. Furthermore, aptamer 19 significantly increased the survival rate of mice with endotoxemia. The results suggest that a novel LPS antagonizing aptamer was obtained by SELEX, which successfully treated experimental sepsis.

Introduction

Sepsis is an important cause of death for critical patients. However, no specific treatment for sepsis is available [1–5]. In a clinical setting, more than 50% of sepsis cases are caused by Gram-negative bacteria [6,7]. Gram-negative bacterial endotoxins (a compound glycolipid called LPS) are one of the major causes for sepsis. After LPS binds to lipopolysaccharide binding protein (LBP), it is transferred to membrane receptors, Toll-like receptor 4 (TLR4), MD2, and CD14 complex, causing cell activation, NF-κB activation, and release of large amounts of cytokines including TNF-α and IL-1. Consequently, many inflammatory mediators participate in a cascade, resulting in inflammatory reaction imbalances and ultimately, sepsis, septic shock, and MODS [8–11]. Hence, LPS and endotoxemia are the most important precipitating or initiating factors for Gram-negative bacteria-caused sepsis and septic shock. Therefore, an anti-endotoxin treatment may play an important role in the management of sepsis [12]. More and more researchers have recognized the importance of antagonizing and blocking the endotoxin, and some advances have been achieved [13–16]. Agents targeting LPS and key pathogenic steps leading to sepsis have been shown to have a modest efficacy, but have not been used clinically. Meanwhile, these agents also have drawbacks. Although some agents show potential for clinical application, most were designed using a computer or were synthesized chemically, and consequently may possess low specificity and weak antagonizing action.

SELEX is a novel combinatorial chemistry technology developed in the early 1990s [17]. Its function is to exponentially enrich aptamers that are screened and to specifically bind target molecules using a large-capacity random oligonucleotide library and PCR technology. Moreover, compared with protein or polypeptide drugs, nucleic acid drugs have several advantages because they are highly tissue permeable, not immunogenic, not rapidly degraded *in vivo* and easily stored and shipped. Since its emergence, SELEX has been developed rapidly, and this technology shows a bright outlook for future applications in rapid drug development, clinical diagnosis and treatment, and basic evolutionary research [18–20]. Using this technology, a VEGF aptamer, NX1838, has been used in clinical trials in an attempt to prevent pathologic angiogenesis and has been approved by the FDA for ophthalmologic applications [21].

In this study, aptamers binding specifically to LPS were screened by SELEX. We obtained oligonucleotide aptamers that inhibited LPS-induced toxicity significantly. Moreover, these compounds significantly protected mice with LPS-induced sepsis.

Materials and methods

Materials

LPS (0111B4, Lot No. 69H4157) was purchased from Sigma Co. and used for ssDNA library screening. Taq DNA polymerase, T4

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polynucleotide kinase and DNA extraction kit were purchased from Promega Company. [γ-^{32}P]ATP was purchased from Beijing Yahui Co., Ltd. (Beijing, China). Nitrocellulose membrane (0.45 μm, Millipore), and tRNA and BSA (Huamei Biotech Company, Beijing, China) were stored in our department. Guanidinium isothiocyanate and cell culture supplies were purchased from Gibco Company. DL-2000 DNA Marker was purchased from Takara Company (Japan). The pGMT vectors and DNA purification and recovery kits were purchased from Takara Biotech (Dalian, China).

Construction of ssDNA random library

The ssDNA random library consists of 84 nt random ssDNAs. P84 library: 5′-TAGGGAATTCGTCACGGATCC-N40-CTGCAGGTCGAC GCATGCGCCG-3′, with a constant sequence at either end and a random sequence at the middle N40. The library capacity is approximately 10^{15}. PCR primers: sense 5′-TAGGGAATTCGTCGACG-3′; antisense 5′-CGGCGCATGCGTCGACCTG-3′. The random ssDNAs and primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

Screening of aptamers specifically binding to LPS

Aptamers were screened by the method described previously [22] with slight modifications.

Screening. The constructed ssDNAs (400 pmol) were incubated at 85 °C for 20 min. They were then placed on ice for 5 min, before mixing with 4 μg LPS in 400 μl buffer (concentration 20 mmol/L, pH 7.35, Tris–HCl, 137 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$) followed by 1 h incubation at 37 °C. A 0.45 μm nitrocellulose membrane with a diameter of 2.5 cm was moistened with redistilled water and placed onto a vacuum filter. The mixture was filtered through the nitrocellulose membrane three times. Then the nitrocellulose membrane was cut into pieces and subjected to recovery ssDNA using 7 M urea, phenol or chloroform. A dsDNA library was constructed by gradient PCR amplification of ssDNA. The PCR system consisted of 0.1 μg ssDNA, 10 μl 10× PCR buffer, 6 μl $MgCl_2$, 100 μmol/L dNTPs, 2 U Taq DNA polymerase, 100 pmol sense primer, 100 pmol antisense primer, and brought to a final volume of 100 μl with deionized water. The concentration ratio of sense primer to antisense primer was 1:100. PCRs cycled through 5 min initial denaturation at 94 °C, 40 s denaturation at 94 °C, 1.5 min annealing at 58 °C, 5 min extension at 72 °C, and 10 min final extension at 72 °C. PCR amplification products were subjected to 1.8% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide on a 260 nm fluoroscopic viewing plate. Once the reddish yellow band was obtained, DNA recovery and purification were performed using the kit. Some DNA samples were stored for later use, and the remaining sample was subjected to asymmetric PCR amplification of dsDNA to construct a ssDNA library. The dsDNA was recovered using the recovery kit for the next round of screening. The PCR mixture contained 0.1 μg dsDNA, 10 μl 10× PCR buffer, 6 μl $MgCl_2$, 100 μmol/L dNTPs, 2 U Taq DNA polymerase, 100 pmol sense primer, 100 pmol antisense primer (concentration ratio of sense primer: antisense primer, 1:100) and deionized water combined to a total volume of 100 μl. The conditions of PCR amplification: 5 min initial denaturation at 94 °C, 40 s denaturation at 94 °C, 1.5 min annealing at 58 °C, 5 min extension at 72 °C, and 10 min final extension at 72 °C. The amplified ssDNAs were subjected to screening.

For the 5th to 9th round of screening, the reaction volume was 200 μl, containing 250 pmol random ssDNAs and 2.5 μg LPS. For the 10th to 12th round of screening, the reaction volume was 200 μl, containing 100 pmol random ssDNAs and 0.5 μg LPS. The buffer for these experiments consisted of 20 mmol/L Tris–HCl (pH 7.35), 137 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, and 1 mmol/L $MgCl_2$.

After each round of screening, the binding affinity was measured by radiolabeling to determine the LPS and ssDNA library binding saturation and the number of screening rounds. T4 polynucleotide kinase and [γ-^{32}P]ATP were used to label the primitive ssDNA random library and the screened dsDNA enrichment library. In brief, thermally denatured and radiolabeled ssDNA (final concentration: 5 nmol/μl) was mixed with LPS or BSA (control group, final concentration 300 nmol/μl) and 100 μl buffer (components described for screening), and incubated at 37 °C for 1 h. The reaction system was subjected to vacuum filtration through a nitrocellulose membrane. Then, the nitrocellulose membrane was washed with buffer, and the residual radioactivity was determined using a liquid scintillation counter (Beckman LS 5000). Each sample was analyzed in triplicate.

Cloning and sequencing

ssDNAs obtained after 12 rounds of screening were amplified to dsDNAs by PCR under the same conditions described for screening. dsDNAs were then recovered and purified. The pGMT vector was ligated using the DNA ligation kit according to the kit manufacturer's instructions, and the resulting products were used to transform *Escherichia* coli DH5α following the kit manufacturer's instructions. After Blue-white screening, 50 clones were selected randomly and sequenced using ABI Prism 7000 Automated Sequence Detection System.

Analysis of primary and secondary structures

The primary structure of oligonucleotide aptamers was analyzed using Clustal W software. These aptamers were preliminarily classified according to sequence alignment results and partly conserved sequences. Secondary structure analysis of some sequences was conducted according to Cheng et al. [23] using the FOLD program of GCG software.

Determination of specific binding affinity of aptamers and LPS

According to the structural characteristics, seven aptamer sequences were synthesized, and the binding affinity of aptamers and LPS was determined by radiolabeling. Oligonucleotide aptamers (10 pmol) labeled with [γ-^{32}P]ATP at the 5′ end and 100 ng/ml LPS were mixed with binding buffer (reaction volume 100 μl), and incubated for 1 h at 37 °C. The reaction system was subjected to vacuum filtration through a nitrocellulose membrane. Then, the nitrocellulose membrane was washed with buffer, and residual radioactivity was determined using a liquid scintillation counter (Beckman LS 5000). Each sample was analyzed in triplicate. The relative binding affinity was proportional to radioactivity (CPM).

Functional characterization of high-affinity aptamers: screening LPS-inhibiting aptamers for animal study

Aptamer inhibition of NF-κB activity on LPS-challenged human peripheral blood monocytes. Human peripheral blood monocytes were isolated and cultured as described previously [24]. There were three study groups: stimulation by LPS (100 ng/ml), LPS (100 ng/ml) + DNA aptamer (10 nmol) or LPS (100 ng/ml) + DNA aptamer (20 nmol). The cells were cultured for 6 h, and monocyte NF-κB activity was detected using ELISA detection kit (Active Motif Company, USA) as described previously [24] and according to the manufacturer's instructions.

Effect on IL-1 and TNF-α content of culture supernatants of LPS-challenged human peripheral blood monocytes. The density of human peripheral blood monocytes was adjusted with RPMI 1640 medium containing 5% calf serum to 2×10^6 cells/L. The cells were challenged by LPS (100 ng/ml), LPS (100 ng/ml) + DNA aptamer (10 nmol) or LPS (100 ng/ml) + DNA aptamer (20 nmol), and cultured 6 h. Then, 100 μl of the culture supernatant was removed to determine IL-1 and TNF-α content using an ELISA kit (Jingmei Company, Beijing, China) according to the manufacturer's instructions.

Effect of endotoxemia blockade with aptamer 19 on mouse mortality

Aptamer 19 displayed strong LPS-inhibiting activity and was therefore selected to test the efficacy of an LPS blocker *in vivo*. According to Macagno et al. [25], endotoxemia mouse models can be reproduced by injection of LPS in C57BL/6 mice. In the first group (sepsis; $N = 20$), each mouse was injected via the caudal vein with 1 mg LPS (0111B4). In the second group (LPS + DNA aptamer 19; $N = 20$), each mouse was injected with 1 mg LPS and 10 μmol DNA aptamer. In the third group (LPS + DNA aptamer 18; $N = 20$), each mouse was injected with 1 mg LPS and 10 μmol DNA aptamer. The mice surviving 12 h, 24 h, and 72 h after injection were counted, respectively, and the survival rate was calculated as a percent (number of surviving mice/total number of mice).

Statistical analysis

Experimental data are expressed as mean ± SD. Group differences were analyzed by independent sample *t*-tests using SPSS13.0 software, and a p value <0.05 was deemed statistically significant.

Results

Relative binding rate of ssDNA and LPS

In the control group, BSA alone was used. As shown in Fig. 1A, with increasing rounds of screening, CPM in the BSA group did not change significantly, indicating no significant ssDNA enrichment. In contrast, with increasing rounds of screening, CPM increased significantly in the LPS group. After nine rounds of screening, CPM did not increase significantly, demonstrating a significant enrichment of LPS binding oligonucleotides in the ssDNA library. CPM did not change significantly after 12 rounds of screening, and thus screening was terminated. ssDNAs were recovered by electrophoresis after 1, 3, 5, 7, 9, 10, 11, and 12 rounds of screening. The results demonstrated that with increasing rounds of screening, the purity of the sample was enhanced, as shown by electrophoresis, and became more obvious after 9 rounds of screening (Fig. 1B).

Cloning, sequencing, and structural analysis of oligonucleotide aptamers

Fifty positive clones were selected randomly and sequenced to obtain 46 sequencing results. Analysis demonstrated that the primary structures could be classified into: (1) identical sequences, (2) relatively conserved sequences; and (3) non-conserved sequences (Supplemental Table). There were 21 sequences identical to aptamer 19, and the sequences of aptamers 18 and 19 were highly homologous, with only one base difference. Secondary structure analysis of some sequences of each type demonstrated that secondary structure differed significantly between these sequence types, and was usually a hairpin or stem–loop structure (Supplemental Figure).

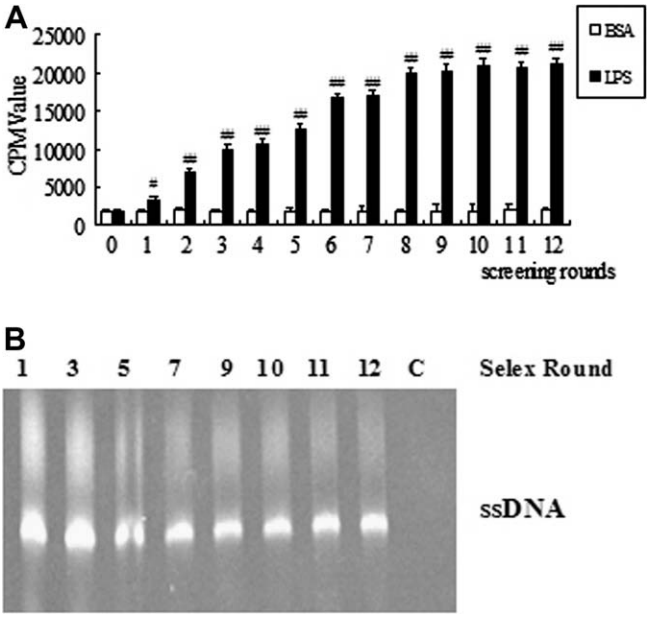

Fig. 1. Relative binding rate of ssDNA and LPS. (A) CPM of residual radioactive nucleic acids on nitrocellulose membrane after screening ($X \pm s$, $n = 3$), $^{\#}p < 0.05$ vs. initial library (0 round), $^{\#\#}p < 0.001$ vs. initial library (0 round). There were no significant differences after nine rounds of screening, demonstrating near saturation. $p > 0.05$: BSA group vs. initial library, demonstrating no ssDNA enrichment. (B) Electrophoregram of screened ssDNA. With increasing screening rounds, the electrophoresis band gradually became a unitary band, particularly after 9–12 rounds of screening, demonstrating that with increasing screening rounds, aptamers with a high affinity for LPS were significantly enriched.

Determination of affinity of specific LPS-binding aptamers and LPS

According to the structural characteristics, the affinity of seven aptamer sequences and LPS was determined by radioimmunoassay (Fig. 2). Various aptamers showed different affinities to LPS, and aptamer 19 had the highest CPM, indicating it displayed the highest affinity. Aptamer 18 was highly homologous to aptamer 19 and also showed high affinity for LPS.

Effect of aptamers on NF-κB activity and IL-1 and TNF-α concentration of culture supernatants of LPS-challenged human peripheral blood monocytes

As shown in Fig. 3A, aptamer 19 most significantly inhibited the NF-κB activity of LPS-challenged human monocytes in a dose-dependent manner. However, aptamer 18 did not inhibit NF-κB activity, but increased the NF-κB activity of LPS-challenged monocytes. In light of high homology of their sequences, we analyzed

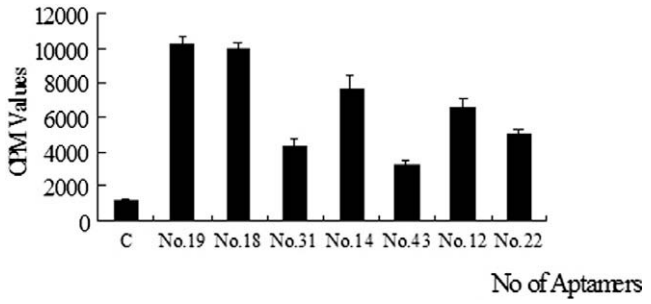

Fig. 2. LPS affinity of some sequences ($n = 3$). The LPS affinity of seven aptamers was determined by radiolabeling, demonstrating the LPS affinity of aptamers 18 and 19 were the highest.

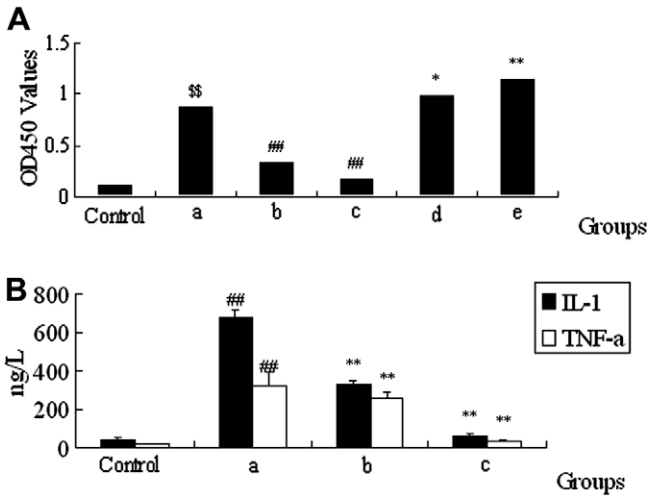

Fig. 3. Effect of aptamers 18 and 19 on NF-κB activity and IL-1 and TNF-α concentration of culture supernatants in LPS-challenged monocytes. (A) NF-κB activity of monocytes was determined by ELISA. Control: not challenged by LPS; a: LPS (100 ng/ml); b: LPS (100 ng/ml) + aptamer 19 (10 nmol); c: LPS (100 ng/ml) + aptamer 19 (20 nmol); d: LPS (100 ng/ml) + aptamer 18 (10 nmol); e: LPS (100 ng/ml) + aptamer 18 (20 nmol). Data are expressed as mean ± SD ($n = 3$). $^{\$\$}p < 0.01$ vs. control group; $^{\#\#}p < 0.01$ vs. group A; $^{*}p < 0.05$ vs. group A; $^{**}p < 0.01$ vs. group A. (B) The concentration of IL-1 and TNF-α of monocytes were determined by ELISA. Control: not challenged by LPS; a: LPS (100 ng/ml); b: LPS (100 ng/ml) + aptamer 19 (10 nmol); c: LPS (100 ng/ml) + aptamer 19 (20 nmol). Data are expressed as mean ± SD ($n = 6$). $^{\#\#}p < 0.01$ vs. control group; $^{**}p < 0.01$ vs. group A.

their secondary structure and demonstrated that their secondary structures differed significantly, which may account for their varied bioactivity (Supplemental Figure: aptamer 18 and aptamer 19). To further confirm the inhibitory effect of aptamer 19 on LPS-challenged monocytes, the effect of aptamer 19 on the IL-1 and TNF-α concentration of culture supernatants of LPS-challenged human monocytes was analyzed by ELISA. As shown in Fig. 3B, aptamer 19 significantly decreased IL-1 and TNF-α content of culture supernatants of LPS-challenged human monocytes in a dose-dependent manner.

Effect of aptamer 19 on the mortality of mice with endotoxemia

Aptamer 19 (10 μmol) significantly decreased the mortality of mice with LPS-induced sepsis, which was apparent 12 h after LPS injection. As shown in Fig. 4, the survival rate of mice treated with aptamer was 75% at 12 h after LPS injection, but was 10% in the control group ($p < 0.05$). At 48 h and 72 h after LPS injection, the survival rate was 60% in mice treated with aptamer 19, and was 0% in the control group ($p < 0.05$). However, aptamer 18 reduced the survival rate of mice at 12 h after LPS challenge, suggesting that aptamer 18 does not protect mice with endotoxemia. The results further demonstrated that aptamer 19 significantly inhibited the bioactivity of endotoxin, and may be a successful endotoxin antagonist for sepsis prevention and treatment.

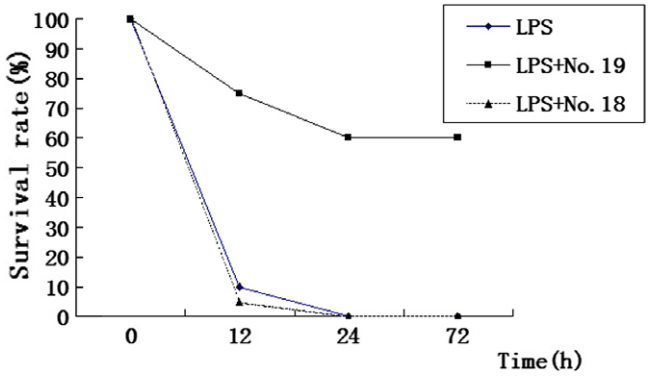

Fig. 4. Aptamer 19 inhibited LPS-induced endotoxin shock in mice. C57BL/6 mice were injected with LPS via caudal vein (1 mg/mouse, $n = 20$), or with LPS and 10 μmol aptamer 19 ($n = 20$) or with LPS and 10 μmol aptamer 18 ($n = 20$). The mice surviving 12 h, 24 h, and 72 h after injection were counted, and the survival rate was calculated (%).

Discussion

In this study, we obtained oligonucleotide aptamers that significantly inhibited LPS activity using novel SELEX technology. Meanwhile, we found that these aptamers significantly increased the survival rate of sepsis model mice. To our knowledge, for the first time we report that LPS activity inhibiting aptamers identified by SELEX significantly protected mice with endotoxemia. Hence, these aptamers have a bright future for various applications, and further investigation may shed light on the prevention and treatment of Gram-negative bacteria-caused sepsis.

Although SELEX can be used to screen a variety of target molecules, specific screening methods should be applied to various target molecules. Classic SELEX adopts liquid phase binding, nitrocellulose membrane separation, and phenol/chloroform recovery to capture high-affinity nucleic acid sequences from random libraries [26]. However, the phenol/chloroform recovery method is complex and seldom used. We found that it difficult to obtain LPS binding nucleic acid sequences through solid phase screening using microplates as supporters. When isolating Raf-1RNA aptamers, Kimoto found it easier to obtain nucleic acid sequences bound to target molecules by liquid phase separation methods [27]. The possible cause for these findings may occur since LPS, as a lipopolysaccharide, binds poorly to microplates. However, after four rounds of liquid phase screening, the affinity of nucleic acids to LPS was enhanced significantly. During solid phase screening, the natural configuration of target molecules may be changed, or the epitopes of target molecules for nucleic acid binding be masked; furthermore, free movement of target molecules may be influenced. Meanwhile, liquid phase separation methods can more effectively retain the natural configuration of target molecules and nucleic acids. In addition, movement, contact and collision of nucleic acids and LPS are more likely to occur in liquid phase than in solid phase.

The sequence and structure of oligonucleotide is closely associated with its bioactivity. We found that and aptamers 18 and 19 displayed a high sequence similarity. Although they both had a high affinity for LPS, their effects on NF-κB activation were opposite, aptamer 19 significantly suppressed the NF-κB activity in monocytes challenged by LPS, while aptamer 18 exhibited an enhancement effect and decreased the survival rate of mice with endotoxemia. Hence, we postulate that the function of aptamers is mainly associated with their secondary structure. Further analysis indicated that they had significantly different secondary structures, which may account for the distinct bioactivity of these oligonucleotide aptamers. Aptamer 19 contains a hairpin structure with a main loop, and aptamer 18 has a stem–loop structure with double loops. Meanwhile, we analyzed the secondary structure of aptamers that have relatively conserved sequences, but are functionally different, and obtained similar results (unpublished data). Therefore, we postulate that the LPS-inhibitory activity of aptamers is mainly associated with their hairpin structure. We did not analyze the secondary structure of other sequences, hence, further study on the structure and function relationship of aptamers will help enhance the understanding of their function and bioactivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.152.

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