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Screening of RNA molecules inhibiting human acetylcholinesterase by virtue of systematic evolution of ligands by exponential enrichment[1]

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KEY WORDS acetylcholinesterase; SELEX; aptamer

ABSTRACT

AIM: To acquire the specific RNA aptamers inhibiting human red blood cell (RBC) acetylcholinesterase (AChE). **METHODS:** Systematic evolution of ligands by exponential enrichment (SELEX) aptamer against human red blood cell membrane AChE was selected by microtiter plate method *in vitro*. The specific binding to AChE was determined by gel mobility shift analysis. Microcolorispectrophotometric method was used to measure the activity of AChE. **RESULTS:** The aptamers to human RBC AChE were indentified by 9 reiterative rounds. At the same concentration (2.25 μmol/L), the aptamers did not bind to the recombinant human butyrylcholinesterase (rhBChE) but specifically bound to human RBC AChE and inhibited the enzyme activity. **CONCLUSION:** It is an effective way to isolate the specific AChE inhibitor from the vast oligonucleotide combinatorial library by virtue of SELEX.

INTRODUCTION

Systematic evolution of ligands by exponential enrichment (SELEX)[1] is an oligonucleotide-based combinatorial chemistry high-tech using for isolation of high-affinity high-specific ligands (aptamer) for a wide variety of macromolecules and low molecular weight targets[2,3]. Specific single-strand oligonucleotides (either RNA or DNA) with high affinity for targets are isolated from huge library of random sequence molecules by reiterative rounds of selection and amplication. Aptamers can be engineered readily to function as either therapeutics or dignostics[4]. For instance, aptamers that bind to human immunodeficiency virus type I integrase or reverse transcriptase can inhibit viral replication[5].

The present study was to acquire aptamers binding to the human acetylcholinesterase (EC 3.1.1.7, AChE) inhibiting enzyme activity. Inhibition of AChE is routinely employed for an increasing variety of clinical applications (eg, Alzheimer's disease drugs, muscle relaxants or prophylactics in protection against nerve agents attack).

MATERIALS AND METHODS

Reagents Human RBC membrane AChE (SDS-PAGE pure, 1.1 g/L) was a gift from Dr LIAO Jian (University of Bern, Switzerland). Microtiter plate was obtained from Nunc Co (Denmark). T7 transcription kit and reverse tanscription kit were purchased from

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Promega (USA). PCR amplification kit was from TakaRa Co (Dalian, China). Single-stranded DNA primers and templates were synthesized by Bioasia Inc (Shanghai, China). [α-^{32}P]UTP was from Yahui Inc (Beijing, China). All other chemicals were of AR grade.

Oligonucleotide library A population of 1×10^{14} DNA templates (G library) each with a 25-nucleotide (25 N) variable region and 5' and 3' fixed regions were created by oligonucleotide synthesis, and a 85-nucleotide double-stranded PCR product of each was constructed.

5' primer containing the T7 promotor underlined:
5'-TAATACGACTCACTATAGCAATGGTACGGTACT-TCC-3';
3'primer:
5'-TTAGCAAAGTAGCGTGCACTTTTG-3'.
ssDNA-G template:
5'-TAATACGACTCACTATAGCAATGGTACGGTACT TCC(25N)CAAAAGTGCACGCTACTTTGCTAA-3';

***In vitro* selection** The RNA pool was transcribed from the amplified DNA template using a T7 *in vitro* transcription kit[6]. After purification on an 8 % denaturing polyacrylamide gel, the RNA pellet was dissolved in sodium phosphate-buffered saline (PBS, pH 7.4).

For selection of aptamers directed to AChE, 9 rounds of enrichement were carried out consecutively[7]. For each SELEX round, microtiter plates were kept at 4 °C overnight with AChE solution in PBS (Tab 1). RNA input into each round was also described in Tab 1. After discarding the supernatant, wells were washed with the SHMCK blocking buffer (Hepes 20 mmol/L, pH 7.35, NaCl 120 mmol/L, KCl 5 mmol/L, $CaCl_2$ 1 mmol/L, $MgCl_2$ 1 mmol/L, and casein 1 g/L) for 6 times. RNA was diluted into 200 μL of binding buffer (SHMCK blocking buffer containing 0.05 % Tween-20), denatured at 95 °C for 5 min, annealled on ice for 5 min, then added into the AChE-coated wells and incubated at 37 °C for 2 h. Following casting off the supernatant and washing with the binding buffer for 6 times, the dry well was placed on the top of a 95 °C-heat block for 5 min. Standard reverse transcriptase reaction (50 μL) were performed at 48 °C directly in the wells, and the reaction products was used for standard PCR and transcription reaction. The 5' and 3' primers were used for template amplication and reverse transcription[6].

Mobility shift assay The binding ability of RNA to AChE was detected by gel mobility shift assay. Radiolabeled RNA samples were acquired by transcription, then heated to 95 °C for 5 min and cooled on ice over 5 min. RNA (1.2 nmol/L) were mixed with AChE in 20 μL total volume of 1×shift buffer (Hepes 10 mmol/L, pH 7.4, KCl 100 mmol/L, dithiothreitol 1 mmol/L, $MgCl_2$ 1 mmol/L, edetic acid 1 mmol/L, tRNA 50 mg/L, and 5 % glycerol). The binding reaction was carried out at 4 °C for 30 min and then fractionated by means of 8 % non-denaturing polyacrylamide gel electrophoresis (29: 1, acrylamide / bisacrylamide, 1×TBE) at 4 °C for 3 h at 80 V. The gel was dried and exposed to autoradiographic film at -20 °C. Mobility shifts of RNA-rhBChE were performed in the same way.

AChE assay Different concentrations of 9-round aptamers (G_9) and the primary RNA library (G_0) in PBS were separately denatured at 95 °C for 5 min and cooled on ice over 5 min, then added AChE solution and incubated at 4 °C for 1 h. The enzyme assay was performed as described previously[8]. In this expriment the normal AChE activity was 5.8 mol· h^{-1}· g^{-1} (protein). The relative AChE activities of the samples were expressed in terms of percentages of the normal enzyme control.

RESULTS

Protein (AChE) and RNA input in each round RNA input in every round was 200 pmol/well. The amount of coated AChE was decreased in the last four rounds in order to increase the stringency of the selection (Tab 1).

Tab 1. Protein and RNA input into each SELEX-round. Both protein (AChE) and RNA were added to each well in a total volume of 200 μL. AChE denoted the amount added to coat the well, it did not represent the total protein adsorbed.

Round	AChE /pmol per well	RNA/pmol per well
1	134	200
2	134	200
3	134	200
4	134	200
5	134	200
6	67	200
7	67	200
8	67	200
9	13.4	200

***In vitro* selection of aptamer binding to AChE** After 9 cycles of selection and amplification, a new

dark band (Fig1, lane d) was clearly observed. The formation of AChE-RNA complex indicated that aptamers directed to the human RBC AChE had been successfully sorted out.

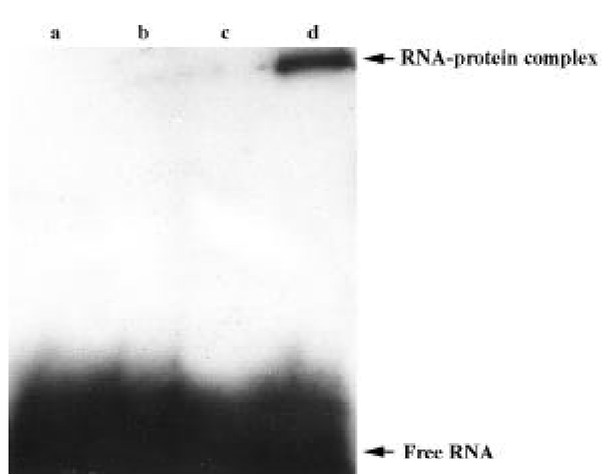

Fig 1. Gel mobility shift assay for G_0 and G_9 RNA. a) G_0 RNA; b) G_0 RNA incubated with AChE 2.25 μmol/L; c) G_9 RNA; d) G_9 RNA incubated with AChE 2.25 μmol/L.

Concentration-dependence of AChE-RNA formation Various concentrations of AChE were reacted with G_9 RNA (1.2 nmol/L). It could be seen that the AChE-RNA complex bands become darker and darker (Fig 2) along with the increasing concentrations of AChE. It suggested that the AChE-RNA complex formation was concentration-dependent.

Specificity of the aptamers Radiolabled aptamers (1.2 nmol/L) reacted well with the hRBC AChE at the concentration of 2.25 μmol/L (Fig 3, lane c), while no binding could be seen in case of rhBChE at the same concentration (Fig 3, lane b). It indicated that the aptamers selected by SELEX were highly specific to human RBC AChE.

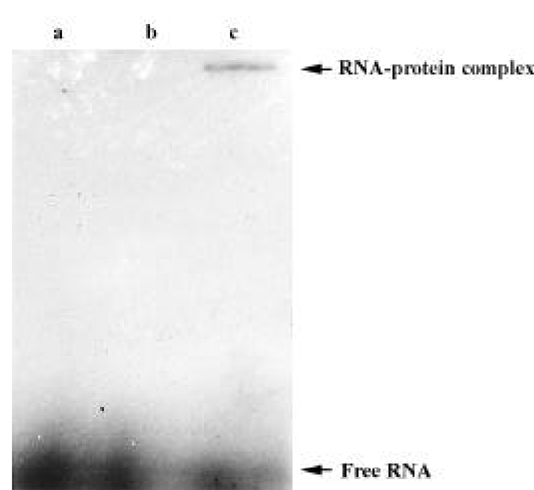

Fig 3. Specificity of G_9 RNA aptamers to human AChE. Gel mobility shift assay was used to examine the protein-aptamer interaction. a) G_9 RNA 1.2 nmol/L; b) G_9 RNA 1.2 nmol/L+rhBChE 2.25 μmol/L; c) G_9 RNA 1.2 nmol/L+hRBC AChE 2.25 μmol/L.

Inhibition of hRBC AChE activity by RNA aptamers AChE activity was inhibited along with the increasing G_9 RNA aptamer concentrations. G_0 RNA did not show any inhibition on AChE activity (Fig 4).

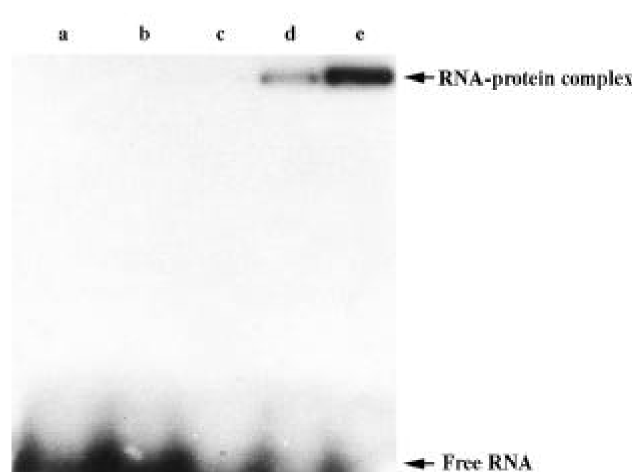

Fig 2. Gel mobility shift assay for G_9 RNA incubated with various concentrations of AChE. a) no protein; b) AChE 0.38 μmol/L; c) AChE 0.75 μmol/L; d) AChE 3 μmol/L; e) AChE 6 μmol/L.

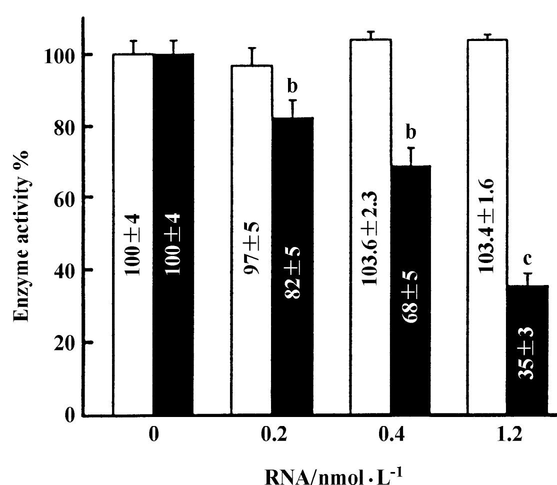

Fig 4. Inhibition of rhRBC AChE activities by G_0 and G_9 RNA aptamers. *n*=3 independent experiments in duplicate. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* G_9 aptamer control. Open boxes: G_0 aptamers; Black boxes: G_9 aptamers.

DISCUSSION

In this paper, we used *in vitro* selection and amplification to identify the RNA aptamers that bind to the human RBC AChE.

The critical step in SELEX is how to sort out the aptamer-target complex from the reaction mixture. There are several ways to separate aptamer-target complex from unbound fractions. Using a 96-well microtiter plate coated with the protein target is the simplest way in trapping of the complex. It lays out the basis for the high thoughput SELEX[6].

Aptamer-based diagnostic reagents rivaling antibodies may be used in clinic owing to the high affinity and specifity of aptamer[9]. Aptamers directed to AChE can be used in immunohistochemistry and Western blotting for AChE detection[10].

The amino acid sequence of AChE in nervous system is distinct from that on the red blood cell membrane only at the C-terminus of the enzyme, the last 43 residues. The aptamers inhibiting hRBC AChE will inhibit neuronal AChE since they share an identical active center. In view of that anti-cholinesterases have been the first choice in the therapy of Alzheimer's disease and Myasthenia gravis[11,12], the specific aptamers to AChE will be used as therapeutic or dignostic agents in the future.

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REFERENCES

1 Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990; 249: 505-10.

2 Gold L. Oligonucleotides as research diagnostic and therapeutic agents. J Biol Chem 1995; 270: 13581-4.

3 Butz K, Denk C, Fitscher B, Crnkovic-Mertens I, Ullmann A, Schroder CH, *et al*. Peptide aptamers targeting the hepatitis B virus core protein: a new class of molecules with antiviral activity. Oncogene 2001; 20: 6579-86.

4 Brody EN, Gold L. Aptamers as therapeutic and diagnostic agents. J Biotechnol 2000; 74: 5-13.

5 Tarrago-Litvak L, Andreola ML, Fournier M, Nevinsky GA, Parissi V, Soultrait VR, *et al*. Inhibitors of HIV-1 reverse transcriptase and integrase: classical and emerging therapeutical approaches. Curr Pharm Des 2002; 8: 595-614.

6 Fitzwater T, Polisky B. A SELEX primer. Methods Enzymol 1996; 267: 275-301.

7 Drolet DW, Jenison RD, Smith DE, Pratt D, Hicke BJ. A high throughput platform for symstematic evolution of ligands by exponential enrichment (SELEX). Comb Chem High Throughput Screen 1999; 2: 271-8.

8 Li FZ, Sun MJ. Microcolorimetrically method of hydroxylamine to measure the acetylcholinesterase activity. Bull Acad Mil Med Sci 1986; 10: 211-4.

9 Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin Chem 1999; 45: 1628-50.

10 Bianchini M, Radrizzani M, Brocardo MG, Reyes GB, Gonzalez Solveyra C, Santa-Coloma TA. Specific oligobodies against ERK-2 that recognize both the native and the denatured state of the protein. J Immunol Methods 2001; 252: 191-7.

11 Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H. Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem 1999; 264: 672-86.

12 Soreq H, Glick D. Novel roles for cholinesterase in stress and inhibitor response. In: Giacobini E, editor. Cholinesterase and cholinesterase inhibitors. London: Martin Dunitz; 2000. p 47-61.