Published in final edited form as:

Appl Biochem Biotechnol. 2016 September ; 180(1): 10–25. doi:10.1007/s12010-016-2081-0.

Selection of RNA aptamers against botulinum neurotoxin type A light chain through a non-radioactive approach

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Abstract

Botulinum neurotoxin (BoNT), a category A agent, is the most toxic molecule known to mankind. The endopeptidase activity of light chain domain of BoNT is the cause for the inhibition of the neurotransmitter release and the flaccid paralysis that leads to lethality in botulism. Currently, antidotes are not available to reverse the flaccid paralysis caused by BoNT. In the present study, a non-radioactive based SELEX process is developed by utilizing surface plasmon resonance to monitor the binding enrichment. Two RNA aptamers have been identified as strong binders against light chain of botulinum neurotoxin type A. These two aptamers showed strong inhibition activity on LCA, with IC₅₀ in nM range. Inhibition kinetic studies reveal mid nanomolar K_I and non-competitive nature of their inhibition, suggesting they have strong potential as antidotes that can reverse the symptom caused by BoNT/A. More importantly, we observed that 2'-fluorine-pyrimidines modified RNA aptamers identified here do not change their binding and biological activities. This observation could lead to a cost-effective way for Systematic Evolution of Ligands by EXponential enrichment (SELEX), by using regular nucleotide during SELEX, and 2'-fluorine-pyrimidines modified nucleotide for final application to enhance their RNase-resistance.

Keywords

RNA; aptamer; 2'-fluorine-pyrimidine; surface plasmon resonance; botulinum neurotoxin; antidote; endopeptidase

Introduction

Botulinum neurotoxins (BoNT) are the most poisonous substances known (1,2). BoNTs produced by anaerobic *Clostridium botulinum* are the cause of botulism, a life-threatening neuromuscular disease. There are currently seven known serotypes of BoNTs, designated as types A to G. More recently, the eighth serotype, type H, has been identified (3), and it is believed that BoNT/H is a hybrid of known serotypes of A and F (4, 5). Serotypes A, B, E,

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and occasionally F have been shown to cause human botulism, and among them, type A is the most potent and has the longest paralysis effect *in vivo* (up to 6 months) (1, 2). An extrapolation from primate studies estimated the median lethal dose (LD₅₀) of type A botulinum neurotoxin (BoNT/A) for an average human weighing 70 kg through intravenous, inhalation and oral routes to be 0.7–0.9 µg, 0.09–0.15 µg and 70 µg respectively (1). Despite significant research efforts and substantial fiscal investment, there is still no effective antidote available, except the equine antitoxin sera, and no safe prophylaxis against botulism (6, 7). Furthermore, there are no rapid detection assays and diagnostic tools for early diagnosis of botulism. The only approved method for detection of BoNT is mouse bioassay, which will take up to 96 hours, and use up to 48 mice (8, 9). Botulism is a rare disease. For example, in 2013, there are only 153 laboratory-confirmed cases in US (10). However, due to their high toxicity, ease of production and dissemination, no effective therapeutics, and no rapid detection methods, BoNTs create utmost fear among the population concerned with bio-terror agents (1, 11, 12) and as a result, BoNTs are classified as “Category A Priority Agents” on National Institute of Allergy and Infectious Diseases (NIAID)’s priority agents (13), and Tier One agents on Select Agent Program (14). BoNTs are the only protein toxin on category A agent list, and Tier One select agent list (13, 14). Taking together, there is an urgent need to develop both therapeutic (including prophylactic) agents against BoNT and develop rapid reliable detection system for BoNTs.

BoNTs are 150 kDa proteins with comprised three functional domains: The light chains (LCs) of neurotoxins are zinc-endopeptidase, which cleave several proteins involved in synaptic vesicle docking and fusion, and therefore, block the release of acetylcholine (1, 2). The heavy chain (HC) plays an accessory role of binding to the target nerve cells (through its C-terminus) and translocation of the LC into the cell cytoplasm (through its N-terminus) (1, 2, 15, 16). Because of the central role of LC during the toxication of BoNTs, it is a valid target for development of both therapeutics and rapid detection. Currently, small molecule inhibitors and antibodies are two main families of antidotes in development against BoNT. Antibodies can only neutralize toxin at extracellular level, and therefore, only have very short treatment window; once symptoms of botulism are developed, antibody-based antidotes are not effective, as they cannot get into the intoxicated neuronal cells (6, 7). Small molecule inhibitors have the potential to be effective antidotes to reverse the paralysis caused by botulism. However, despite great efforts on the development of small molecule based inhibitors, no promising leads have been identified in animal models. One main obstacle for development of small molecule based inhibitors against BoNT is the flexible structure of BoNT in solution, which are somehow different from crystal structures, and pose great challenge for rational design of effective small molecule inhibitors (6, 7).

Aptamers are single stranded oligonucleotides (either DNA or RNA) that form unique three dimensional structures which provide the basis for high binding specificity and affinity towards their targets. Identification of suitable aptamer sequences and thereby structures is typically achieved through a screening process which is popularly termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) (17). This powerful approach has been described in numerous previous reports and therefore will not be detailed herein (17–21). SELEX theoretically has the potential to develop aptamers against any known molecule (17–24) including small molecules (18, 19), proteins (19), cell surfaces (21, 23),

and whole organisms (24) by utilizing a rapid and entirely *in vitro* process. Due to the specificity of these interactions, aptamers may also serve as valuable tools to modulate or block functions of targets. Additional advantages of aptamers include low toxicity, non-immunogenicity, better tissue penetration (due to their smaller size), favorable pharmacokinetics profile (easy to be modified as an on-demand format to fit in different needs), established quality control (through chemical synthesis), and simple storage conditions (6, 19, 25). All these render aptamers an attractive candidate for therapeutic and diagnostic platforms that rival, and in some cases, surpass antibodies. In term of search of effective antidotes against BoNT, SELEX approach is carried out in solution, and therefore, aptamers will directly against the solution structure of BoNT and generate better inhibitor candidates. In addition, aptamers selected from SELEX approach could also be better candidates for detection and diagnosis of botulism, due to their structures directly against native BoNT structures in solution (25, 26). Traditional SELEX involves using radioactive isotope to monitor the enrichment of binding (17). This approach, while being successful, requires radioactive materials, which poses serious health risk for lab personnel and need special protection equipment. When combined with highly biohazard agents such as BoNTs, radioactive based SELEX approach will require even higher safety protections. Therefore, development of non-radioactive SELEX protocol is desirable for selection of highly specific aptamers.

In our earlier studies, by using radioactive based SELEX approach, we have successfully identified three 2'-fluoro-pyrimidines modified RNA aptamers against light chain of botulinum neurotoxin type A (LCA) (27), which not only showed high binding affinity and high specificity to LCA, but also showed strong inhibition effects on enzymatic activity of LCA, and in vivo protection of BoNT intoxication (28). Surface plasmon resonance (SPR) has been developed as an alternative for selection of aptamers (29, 30). However, most current SPR-based SELEX protocols involve immobilize the ligand on the chip-surface (29, 30), which could change structures of ligands. In this study, we developed a non-radioactive SELEX protocol by carrying out the SELEX in solution, while employing SPR technology to monitor the progress of enrichment during SELEX. This approach ensures the aptamers selected still targeting the solution structure of BoNT. By applying this approach, we identified two RNA aptamers, with binding affinity (in term of dissociation constant) below 100 nM. Those two aptamers also showed strong inhibition effect on endopeptidase activity of LCA, with IC₅₀ below 1 μ M. More importantly, while the pools were generated using non-modified nucleotides, when final monoclonal RNA aptamers were incorporated with 2'-Fluoro pyrimidines, both binding affinities and enzymatic inhibition on LCA were remained, suggesting that modified pyridines do not change overall RNA folding, and provide an economic way to select RNA aptamers, by using non-modified nucleotides during selection process and identifying the monoclonal sequence of aptamers, and incorporating modified pyrimidines to produce aptamer in order to improve the chemical stability of RNA aptamers for their applications.

Materials and methods

Recombinant LCA

Recombinant LCA was produced and purified as described previously (31). The enzyme concentration was determined using extinction coefficient of $0.83 \text{ (mg/mL)}^{-1} \text{ cm}^{-1}$ at 280 nm (31). The LCA (with 20% glycerol) was stored at -80°C , and endopeptidase activity was verified prior to screening.

Construction of single strand (ss) DNA random library

The ssDNA random library (5'S24-(R40)-3'S25) consists of a 40 nucleotide random sequence domain which is flanked by constant 5'- and 3'-ends for amplification reactions (5'-CTATAGGGTACCCACTCAGGTACG-(N40)-CAGCTTTCTAGAATTAAGCTTAGGC-3'). Primers used for SELEX are: forward primer (SEL-FWD45 (45-mer)): 5'-CGGCGAATTCTAATACGACTCACTATAGGGTACCCACTCAGGTAC-3'; reverse primer (SEL-REV24 (24-mer)): 5'-GCCTAAGCTTAATTCTAGAAAGCT-3'. T7 polymerase promoter sequence was added to the 5' primer for *in vitro* transcription by T7 polymerase. The ssDNA library and primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Molecular biology reagents for SELEX

dNTP mix and Platinum Taq polymerase High fidelity (Invitrogen, Carlsbad, CA) were used for PCR. ThermoScript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) was used for reverse transcription PCR (RT-PCR). MEGA shortscript High Yield Transcription Kit (Ambion, Austin, TX) was used to produce regular RNA aptamers, while DuraScribe® T7 Transcription Kit (Epicentre, Madison, WI) was used for produce 2'-F-pryrindine modified aptamers.

Surface plasmon resonance binding reagents

Biacore CM5 chips, 10 x HBS-P buffer, non-ionic surfactant P-20 were purchased from GE Healthcare Life Sciences (Piscataway, NJ). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Thermo Scientific (Rockford, IL). Sodium hydroxide and ethylene diamine were obtained from Sigma Aldrich (St. Louis, MO). Molecular biology grade dithiothreitol (DTT) was obtained from Promega (Madison, WI).

Generation of RNA library

In order to perform *in vitro* selection with the randomized RNA library and obtain the most representative pool, an 89-nucleotide DNA sequence (5'S24-(R40)-3'S25) containing 40 random nucleotide sequences was amplified using primers through Taq PCR reaction (Invitrogen, Carlsbad, CA), and converted into double stranded DNA (111-mer). T7 polymerase promoter sequence was added to the 5' end of the forward primer for *in vitro* transcription by T7 polymerase using MEGA shortscript T7 Kit (Ambion, Austin, TX). The active single stranded (84-mer) RNA transcription product was generated by *in vitro* T7

transcription, and was purified through 12% native polyacrylamide gel electrophoresis as the library for SELEX (ss-84 RNA library).

Two-Stage PCR

The forward primer has quite different T_m value from reverse primer. In addition, the reverse primer is about half the length of forward primer and it is very sticky to the ss-89 template. To overcome this problem, a higher annealing temperature was utilized to remove those non-specific bindings. On the other hand, the forward primer is much longer and therefore become fragile and sensitive to high annealing temperature. Thus a two-stage PCR protocol was developed to satisfy these two different requirements. The two-stage PCR protocol has outlined in the schematic diagram of Figure 1.

SELEX

The SELEX was carried out using filter paper binding process to ensure the binding was under native solution environment. The nitrocellular filter paper (0.45 μM , BA85, Sigma, St. Louise, MO) was used for SELEX, as it only has the affinity to protein-RNA complex, but not free RNA, allowing protein-RNA complex retained on the filter paper, while free RNA were washed away.

The SELEX was started by incubating the LCA with ss-84 RNA library. ss-84 Library was first heated to 85°C for 5 minutes, then let it slowly cool down under room temperature for at least 30 minutes. The binding buffer used was phosphate buffered saline (PBS), pH 7.5, with 5mM MgCl_2 , 5 mM DTT, 4 unit/ μL RNaseOut (Invitrogen). Negative SELEX was carried out first by passing ss-84 alone through filter paper. Only RNAs passed through filter paper were collected and amplified for SELEX against LCA. The negative SELEX was used to select RNA that are bound specifically to LCA, but not to filter paper. The initial two round of binding molar ratio of LCA and RNA was 6:1 (1st and 2nd round). The LCA/RNA ratio was gradually decreased to 1:1 (3rd through 5th rounds), 1:5 (6th and 7th round), 1:10 (8th round), 1:20 (9th round), and 1:50 (10th round). The reaction volume was kept at 550 μL , and the binding was carried out at 37 °C for 30 min, followed by filtering through filter paper. The RNA-protein complex retained on filter paper was extracted using 500 μL extraction buffer (PBS, 10 mM EDTA, 0.1% SDS). The extracted RNA from protein-RNA complex then went through phenol-chloroform extraction and alcohol precipitation to separate from protein, and reverse transcriptase PCR (RT-PCR) was carried out using ThermoScript Reverse Transcriptase Kit (Invitrogen) to generate ss-DNA, followed by two-stage PCR and in vitro transcription to generate RNA pool for next round SELEX. All binding and SELEX experiments were carried out in an RNase free environment.

Assessing binding enrichment through surface plasmon resonance (SPR)

Biacore T100 (GE Healthcare Life Sciences, NJ) was used for monitoring the binding enrichment during SELEX. LCA was covalently immobilized to the commercially available CM5 chip surface (GE Healthcare Life Sciences, NJ) *via* a standard amine coupling procedure. LCA was reduced to monomer with 10mM DTT for 1 hr at room temperature before immobilization. The carboxyl groups on the dextrans of the sensor chip were activated with a mixture of 0.4M EDC and 0.1M NHS to form reactive succinimide esters.

Protein ligands containing primary amine groups were then injected over the surface to react spontaneously with the surface ester in order to covalently link to the dextran matrix. 7636.9 pg/mm² of LCA was immobilized on the chip surface. Excess reactive esters were deactivated using ethanolamine. A blank channel activated in a similar manner and capped with ethanolamine was used as negative control. The RNA pools stocks stored at -80° C were thawed and heated to 90° C for 5 mins, followed by slowly cooling to room temperature. With 1x HBS-P (HEPES buffered saline containing 0.5% P-20) as running buffer, each analyte (RNA pool, 100 µg/ml) was injected at 30 µl/min for 2 mins followed by a 300 sec dissociation phase. After every analyte injection, the ligand surface was regenerated with a 30 sec pulse of 50 mM NaOH. Binding analysis was performed by maintaining the chip at room temperature.

Cloning and sequencing

To identify monoclonal sequences of aptamers, TOPO/TA cloning kit (Invitrogen) was utilized to subclone RNA aptamer candidates obtained from the 10th SELEX pool. The X-Gal+LB-Agar plates were applied to implement the blue white test to rapidly examine the success of cloning. The Taq PCR products (ds-111 DNA fragments) from pool-10 were incubated with TOPO/TA vector and reaction buffer under room temperature for 30 minutes, then quickly transformed into top-10 competent cell by heat shock procedure. The PCR product is only in 111-mer length therefore it might be too short to be able to completely disable the function of LacZ operon to stop expressing β-galactosidase. There also could be some leak expressions to form some pseudo-negative colonies with blue color on the plate. In order to solve those false-negative issues, after TOPO/TA cloning, the rapid PCR technique was applied to confirm if the picked colonies contained the inserts (aptamer) or not. Following the confirmation of correct-sized inserts from rapid PCR assay, the plasmid DNA samples purified from those picked colonies, and were sequenced (Genewiz, CA).

Aptamer preparation

The monoclonal aptamers were transcribed from their ds-DNA templates (synthesized by Integrated DNA Technologies, Coralville, IA). Non-modified aptamers was prepared using MEGA shortscript T7 Kit (Ambion, Austin, TX), while 2'-fluorine-pyrimidines modified aptamers were transcribed from their corresponding ssDNA templates using DuraScribe® T7 Transcription Kit (Epicentre, WI).

Binding affinity between aptamers and LCA

Biacore T100 was used to study and compare the binding kinetics of the monoclonal aptamer sequences with modified or unmodified pyrimidines. 3'-End of the purified RNA were specifically labeled with biotin-16-ddUTP using a template independent recombinant terminal transferase enzyme (Roche Applied Sciences Indianapolis, IN) (25).

In order to prepare ligand (aptamer) surfaces, streptavidin was covalently immobilized to the commercially available CM5 chip surface *via* a standard amine coupling procedure (25, 32). After removing unbound streptavidin, 45 RU of the 3'-end biotinylated aptamers were then captured on the biotin binding sites on the streptavidin surface. Flow channel containing streptavidin alone was used as negative control to correct for any refractive index changes

and systematic noise. 1x HBS containing 0.2% P-20 and 1mM DTT was used as running buffer. LCA was first reduced to monomeric units using 10 mM DTT, followed by buffer exchange, and diluted in the running buffer. Various concentrations (0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 nM) of reduced BoNT/A LC were diluted in the running buffer and stored in sample compartment maintained at 10°C until injection. Binding analysis was performed by maintaining the chip at 26° C. Analytes were injected at 30 µl/min for 2 mins followed by a dissociation phase of 300 sec. Each analyte injection was followed by 30 sec pulses of regeneration buffer (12.5 mM NaOH and 2% ethanol) and running buffer, respectively. Binding sensorgrams obtained were further analyzed using the BIAEvaluation software (GE Healthcare Life Sciences, NJ) by using 1:1 binding kinetics model with local Rmax.

Enzymatic inhibition assay against LCA

To further evaluate inhibition effects of identified aptamers, a fluorescence resonance energy transfer (FRET) based LCA endopeptidase assay was conducted with a 13 amino acid peptide substrate (truncated from SNAP-25 (31). The FRET peptide was synthesized by New England Peptide (Gardener, MA) and possessed a purity > than 95% (31). Peptide substrate was dissolved in distilled water as 10 µM stock solution, and LCA were diluted with 20 mM HEPES, pH 8.0, containing 1.25 mM dithiothreitol (DTT) and 0.1% Tween 20 (assay buffer). The assay was carried out by pipetting assay buffer, LCA stock solution (in assay buffer) and 25.0 µL of substrate stock solution (in distilled water) into 96-well black half volume microplates (Corning, Corning, NY). The final concentration of LCA is 50 nM, the final concentration of the substrate is 5 µM, and the final buffer strength is half of the assay buffer. For inhibition assay, LCA and aptamer with the desired concentration(s) were incubated at 37 °C for 30 min before adding the substrate. The substrate-enzyme mixtures were incubated at 37 °C for 4 hours before fluorescence reading. The plates were read using a Molecular Devices M5 fluorescence microplate reader (Sunnyvale, CA), with the excitation wavelength of 490 nm and emission wavelength of 523 nm. Concentration response curves of aptamers were performed using peptide based substrate, and the half maximal inhibitory concentration (IC₅₀) was interplotted from the concentration response curve using a non-linear polynomial regression.

Enzyme kinetics measurements were carried out using the same procedure as outlined above. Substrate concentrations from 5 to 25 µM were used (e.g., 5, 10, 20, and 25µM) for enzyme activity with 25 nM of LCA. The reactions were carried out at 37 °C, with monitoring of fluorescence in the first 10 min to calculate the initial reaction velocity. The fluorescence signals observed were within the linear range for the substrate concentrations chosen above. To evaluate the inhibition kinetics, a given concentration of the aptamer was pre-incubated with LCA at 37 °C for 30 min before adding the substrate. The concentrations of inhibitor used were 300 and 600 nM (final concentration after adding the substrate). All the results shown are the average of six replicates from two different plates (triplicates of each reaction on each plate). Inhibition constant was calculated using the Lineweaver-Burk plot in the presence of the inhibitor, where the slope equals to $\alpha \cdot K_M / V_{max}$, and y-intercept equals to α' / V_{max} . The K_I and K_I' were calculated from α and α' respectively: $\alpha = 1 + [I] / K_I$; $\alpha' = 1 + [I] / K_I'$.

Results and Discussions

SELEX screening and pools examinations

Due to the difference in melting temperatures of forward and reverse primers, the initial PCR products of the library showed higher contents of impurities with length different from that of the desired PCR product (data not shown). The analysis of PCR products indicated that reverse primer still attached to ss-89 template even at 70 °C (data not shown). Considering the length of reverse primer which was just half the length of forward primer, and the stickiness of reverse primer to the template, a higher annealing temperature was utilized to remove the non-specifically bindings of reverse primers. Forward primer, however, is much longer and thus sensitive to higher annealing temperatures. To accommodate of both reverse and forward primers, a two-stage PCR protocol was developed (Fig. 1).

The two-stage PCR protocol successfully removed any impurity bands that appeared in the high molecular weight region of the gel and greatly improved the yield of correct target-band (ds-111 band). Figure 2 demonstrates the effectiveness of two-stage PCR protocol (clear background in high molecular weight region).

During SELEX, we introduce a negative selection by just passing through the RNA library through the nitrocellular filter paper to remove those RNA species that bind to filter paper. This process removed the bias for the later SELEX process, and ensured only RNA-protein complex retained on the filter paper. The selection stringency was controlled by changing the ratio between LCA and RNA (Table 1). The initial two rounds of selection used a much less stringent condition, the ratio of LCA to RNA was 6:1. This less stringent selection allowed enough RNA bound to target during the initial selection. The binding stringency was gradually increased by decreasing the ratio of LCA to RNA in the later selection cycles, to ensure strong binders identified from selection process.

The traditional SELEX protocol uses the radioactive isotope labeled nucleotide to monitor the enrichment during selection process. This will raise safety concerns among lab workers. Here, we developed a non-radioactive SELEX protocol by employing surface plasmon resonance (SPR)-based technology to monitor the enrichment during SELEX. SPR is a label free technology for real-time detection of the binding between target and ligand. To monitor the binding enrichment through each round of SELEX, LCA was immobilized on the BiaCore chip through its amines. RNA pool was flow through the chip surface to monitor the binding between LCA and RNAs. Figure 3 showed that the binding increases as SELEX progressing, suggesting SPR technology is a valid alternative for traditional radioactive based method to monitor the binding enrichment during SELEX. As shown in Fig. 3, the binding reached plateau after six-round SELEX. Therefore, more stringent conditions were used during next five round SELEX (Table 1). To further confirm the biological activity of RNAs in the pool, we examined the inhibition of LCA endopeptidase activity by RNA pools. As shown in Figure 4, the inhibition activity of RNA pool was increased with the progression of SELEX, suggesting that the SELEX protocol developed in this report is a valid approach.

After 11th round of SELEX, the pool-ten RNA was subjected for subcloning to identify the monoclonal sequences. Sequencing revealed 4 sequences out of 69 validated sequences from 100 random colonies chosen (Table 2). TW1 is dominated, and TW 2 and TW 4 only have one occurrence respectively, therefore, further characterizations were performed only on TW 1 and TW 3. The sequences of TW 1 and TW3 aptamers are listed in Table 3. While the SELEX protocol developed here was using non-modified nucleotides, to compare the effect of 2'-fluorine-pyrimidines on the binding and biological activity of aptamers, we transcribed the aptamers using both non-modified nucleotides (non-modified aptamers), and 2'-fluorine-pyrimidines (2'-F modified aptamers).

Binding affinity of aptamers and LCA

SPR based technology was used to further characterize the aptamer binding affinity. In an effort to investigate the effects of 2'-fluorine-pyrimidines on the property of aptamers, both non-modified and 2'-F modified aptamers were used.

LCA binding to the same amount (2 femtomoles) of modified or unmodified aptamers captured on streptavidin surfaces are shown in Figure 5. Both TW1 and TW3 with modified pyrimidines show higher magnitude of binding compared to their unmodified counterparts. Binding sensorgrams obtained for TW1 unmodified aptamer displayed the least magnitude of affinity (Fig. 5). Although RNase free environment and reagents were used during the production of unmodified RNA aptamers, these conditions were technically difficult to maintain during the SPR binding studies. The buffer containers and buffers were maintained RNase-free, while these conditions could not be ensured for the binding surface and microfluidics of the instrument. Thus, the decrease in the binding strength of the unmodified RNA could be attributed to their possible degradation by RNase. Research has shown that replacement of 2'-OH in ribo-sugars of RNA with the highly electro-negative fluorine often resulted in locking the sugars in a C3'-endo conformation leading to significant increase in target affinity in addition to imparting nuclease resistance (33). An increase in target affinity owing to changes in sugar conformation may also contribute to the better binding of modified aptamers. However, the increase in the binding affinity of both the aptamers with a parallel increase in association and dissociation rates observed for the modified aptamers indicate that this increase in affinity may be due to the enhanced chemical stability provided by the 2'-F pyrimidines.

A 1:1 binding model between LCA and aptamer with using local R_{max} was best fitted to the sensorgrams. The equilibrium binding constants, association and dissociation rates were obtained by applying the 1:1 binding model from a series of 8 concentrations of analytes (from 7.8 nM to 1000 nM). The results clearly suggested that the modified TW1 and TW3 aptamers display similar K_D for BoNT/A LC (Table 4). Of the two aptamers, 2'-fluoro-pyrimidines modified TW1 aptamer shows almost twice higher affinity (faster association) accompanied by stronger binding (slower dissociation) compared to that of TW3. Similar trend is also observed in non-modified TW1 and TW3 aptamers, especially for dissociation constants. Despite the significant differences observed in association rates, both modified and unmodified RNA aptamers display nM affinity for their target (LCA) (Table 4).

Inhibition of endopeptidase activity of botulinum neurotoxin type A light chain

The endopeptidase inhibitory activity of TW1 and TW 3 was also investigated. Due to the potential degradation by RNase as observed in binding affinity study, both regular and 2'-F pyrimidines modified RNA aptamers were produced. As shown in Figure 6, both TW 1 and TW 3 showed inhibition on the endopeptidase activity of LCA, however, the non-modified aptamers inhibition activity decreased dramatically after 60 min, suggesting the potential degradation of regular RNA during inhibition assay. Therefore, 2'-F pyrimidines modified RNA aptamers were used for rest of inhibition studies. The inhibition of aptamers on enzymatic activity of LCA is dose-dependent (Figure 7), and the IC_{50} values of these two aptamers are in high nanomolar range (Table 5), with 639 nM for TW 1, and 727 nM for TW3. Considering the concentration of LCA used during inhibition assay (50 nM), the 50% LCA inhibition is achieved when the inhibitor:LCA ratio is 12.8:1 for TW 1, and 14.5 for TW 3. As a control, the inhibition effect of pool 0 RNA did not showed any inhibition activity at 20 μ M. These data demonstrate that inhibition is not due to non-specific interactions between LCA and RNA molecules.

To understand the mechanism of inhibition, we carried out enzyme kinetic studies on this two aptamers. In the absence of the aptamer, the K_M is 22 μ M ($\pm 2 \mu$ M), which is similar to our previously reported value (27, 31). Both aptamers depict a non-competitive inhibition mechanism from the Lineweaver-Burk plot (Figure 8), with different intercepts at Y axis (yet not being parallel) compared to that in the absence of the aptamer. As shown in Table 5, the K_I of TW1 and TW3 aptamers is in mid-400 nM range (433 nM for TW1, and 463 nM for TW3), while the K_I' is 720 nM for TW 1 and 849 nM for TW3. The closeness of K_I and K_I' for both TW1 and TW3 aptamers suggested that these two aptamers are in a classic non-competitive mode, in which the inhibition activity of TW1 and TW3 are due to the binding between aptamers and the enzyme, as well as the binding between aptamers and the substrate-enzyme complex. While non-competitive inhibitors may suggest the binding to an alternative site to enzymes, they could also be active-site binders, such as those enzymes using exosites for substrate binding (34). In case of botulinum neurotoxin, it is known that BoNT/A light chain has two exosites, leading to specific recognition of its substrate, SNAP-25 (35). Aptamers in this study are identified from interaction to LCA in solution, and thus interact with the overall light chain structure. The inhibition of the enzymatic activity of LCA could arise from the direct interaction with the active site of LCA, while the exosite-binding to substrate leads to the non-competitive nature (34). While most drugs on the market or in clinical studies are competitive inhibitors, there are non-competitive inhibitor drugs (34). Fewer noncompetitive inhibitor drugs on the market and in the clinical development reflect the fact of the traditional drug discovery focusing on active-site directed inhibitors. With the application of high throughput approach, more noncompetitive inhibitors will emerge. The non-competitive inhibitors open a greater diversity of inhibitor modalities, which not only include binding to allosteric sites, but also to active sites.

Conclusions

In this report, we successfully developed a non-radioactive SELEX procedure, by employing surface plasmon resonance as primary tool to monitor the binding enrichment during

SELEX. Two RNA aptamers are identified against LCA. These two aptamers showed strong binding affinity to their target (LCA), with dissociation constant being 31.4 nM for TW1 aptamer, and 27.9 nM for TW3 aptamer. In addition, these two aptamers possess strong inhibition effects against endopeptidase activity of LCA. The inhibition behavior of the aptamers is best described as non-competitive inhibition mode. Both IC_{50} and the inhibition constants of those aptamers are in mid-nM range, suggesting that aptamers identified here have the potential to be further developed as therapeutics against the deadly botulism. More importantly, while the SELEX was carried out using non-modified nucleotides, when transcribed with 2'-F-pyrimidine modified nucleotides, the binding affinity, as well as biological activity, are retained. To our best knowledge, this is the first such observation. Our observation is only based on the aptamers we identified, and may not apply to other RNA aptamers. This observation, however, does suggest that the 2'-fluorine-modified pyrimidine may not change the overall structure of RNA, at least for our case, and have the potential to open a more cost-effective way for SELEX.

Acknowledgments

This project is partially funded by an NIH grant (1R21AI070787-01A2) and U.S Army Natick Soldier Center grant (W911QY-09-C-0207). Authors would like to sincerely thank Drs. Ravi Singh and Natalia Singh from Iowa State University, for their help on design the library and SELEX process.

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Figure 1.
The two-stage PCR protocol

Figure 2. PCR results from two-stage protocol. The yield of ds-111 product is improved through two-stage PCR.

Figure 3. SPR binding analysis of SELEX process. LCA was immobilized to a CM5 chip and binding of the heterogeneous RNA pools. Binding increases with each round of enrichment.

Figure 4.

Inhibition of LCA endopeptidase activity by RNA pools from SELEX. Negative control is peptide based substrate only, RNA only is pool 8 RNA without substrate or LCA. Positive control is peptide substrate with LCA. The concentration of peptide substrate was 5 μ M, the concentration of LCA used was 100 nM, and the concentration of RNA pool was 28 μ M.

Figure 5. Comparison of binding between LCA (1.3 μ M) and TW aptamers with 2'-F- modified (m) and unmodified pyrimidines (um).

Figure 6.
The effects of 2'-F-pyrimidine modified and unmodified aptamers on endopeptidase inhibition of LCA.

Figure 7.
Dose-response curve for aptamer-mediated BoNT/A LC endopeptidase inhibition (diamonds for TW1, and triangles for TW3).

Figure 8. Lineweaver-Burk plot of BoNT/A LC inhibition with and without inhibitor aptamers (TW1, panel A; and TW3, panel B). The error bars represent the standard deviation of six measurements.

Table 1

Stringency of SELEX

RNA-pool generated through SELEX	LCA:RNA used during SELEX
Pool-0	No toxin (negative SELEX)
Pool-1	6:1
Pool-2	6:1
Pool-3	1:1
Pool-4	1:1
Pool-5	1:1
Pool-6	1:5
Pool-7	1:5
Pool-8	1:10
Pool-9	1:20
Pool-10	1:50

Table 2

Four sequences revealed from subcloning of pool-10 RNA

Aptamers	TW1	TW2	TW3	TW4
# of colonies	62	1	5	1
% of occurrence	89.8	1.4	7.4	1.4

Table 3
Sequence of aptamers identified from non-radiative SELEX protocol

TW-1 *	5'-GGGUACCCACUCAGGUACG-
	AUUGGUGCGAUAAACUAGACUCGUCAUGCGCAGUCUCCUA-
	CAGCUUUCUAGAAUUAAGCUUAGGC-3'
TW-3 *	5'-GGGUACCCACUCAGGUACG-
	UGGGAUUUGAGCGGGUCACACUAUAAGCACGAGCCUUAGA-
	CAGCUUUCUAGAAUUAAGCUUAGGC-3'

* The highlighted sequence is from random library.

Table 4

Comparison of modified and unmodified TW aptamer binding kinetics

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
TW1 modified	$33.1 \pm 0.1 * 10^{+4}$	$10.4 \pm 1.5 * 10^{-3}$	31.4 ± 4.4
TW1 unmodified	$5.3 \pm 1.4 * 10^{+4}$	$5.8 \pm 1.6 * 10^{-3}$	109.0 ± 9.9
TW3 modified	$16.5 \pm 5.4 * 10^{+4}$	$4.6 \pm 1.6 * 10^{-3}$	27.9 ± 4.3
TW3 unmodified	$4.0 \pm 0.1 * 10^{+4}$	$2.4 \pm 0.3 * 10^{-3}$	59.1 ± 7.3

Table 5IC₅₀ and inhibition kinetics of aptamers

	IC ₅₀ (nM)	IC ₅₀ (Ratio of aptamer to LC)	Inhibition constant K _I (nM)	Inhibition constant K _I ' (nM)
TW1	639 (13) *	12.8	433 (35)**	720 (22)
TW3	727 (12)	14.5	463(60)	849 (76)

* Data in parenthesis are the standard deviation of four measurements.