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In Vitro selection of RNA aptamers that inhibit the activity of type A botulinum neurotoxin

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Abstract

The category A agent, botulinum neurotoxin (BoNT), 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, we have identified three RNA aptamers through SELEX process, which bind strongly to the light chain of type A BoNT (BoNT/A) and inhibit the endopeptidase activity, with IC₅₀ in low nM range. Inhibition kinetic studies reveal low nM K_I and non-competitive nature of their inhibition. Aptamers are unique group of molecules as therapeutics, and this is first report of their development as an antidote against botulism. These data on K_I and IC₅₀ strongly suggest that the aptamers have strong potential as antidotes that can reverse the symptom caused by BoNT/A.

Keywords

RNA; aptamer; botulinum neurotoxin; antidote; endopeptidase

Botulinum neurotoxins, produced by *Clostridia botulinum*, are the most toxic substances known to mankind, the cause of the deadly disease, botulism, and can serve as a bioweapon [1,2]. They are also being used as medical therapeutics, treating an array of neuromuscular diseases, and are used for cosmetic applications [3]. Currently, there are seven known serotypes of BoNT, designated as A to G. Of those, type A, B, E are the most common cause for human botulism, and type A is the most potent among the seven serotypes.

BoNTs are 150 kDa proteins comprised of three functional domains: the light chains (LCs) of neurotoxins are zinc-endopeptidase, which collectively cleave several proteins involved in synaptic vesicle docking and fusion, and therefore, block the release of acetylcholine. The

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heavy chain (HC) plays an accessory role of binding to the target nerve cells (through its C-terminus) and the translocation of the LC into the cell cytoplasm (through its N-terminus) [1, 3].

Current therapy for botulism involves respiratory supportive care and the administration of antitoxin. However, to date there is no treatment that can reverse the flaccid muscle paralysis caused by BoNT after the symptom has developed [2–4]. Because the toxin has widespread medical therapeutic uses for neuromuscular diseases, a massive vaccination is not desirable. Therefore, design of therapeutic agents to reverse flaccid muscle paralysis is the most sought-after therapeutic path. In this regard, light chain of BoNT is the best therapeutic target for treatment of botulism, as its endopeptidase activity is the main biochemical process responsible for the blockage of neurotransmitter release. There have been several efforts to develop small molecule inhibitors of BoNT/A endopeptidase, but such molecules suffer from the possibility of non-specific action under *in vivo* conditions, and provide erratic results under *in vitro* and *in vivo* conditions [5]. Candidates like aptamers with size larger than small molecules yet much smaller than antibodies are likely to provide more consistent results.

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) [6]. SELEX theoretically has the potential to develop aptamers against any known molecule, ranging from small molecules to whole organisms [6–8] 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. All these render aptamers an attractive candidate for therapeutic and diagnostic platforms that rival, and in some cases, surpass antibodies.

In this study, the light chain of BoNT/A (LCA) was used as the target for aptamer selection and development, and the inhibition characteristics of identified aptamers against BoNT's endopeptidase activity were investigated. Three aptamers with high binding affinity and specificity to LCA, and with strong inhibition of endopeptidase activity have been identified. In order to enhance the RNase resistant, we use 2'-Fluoro-pyrimidin modified nucleic acid (2'-F-CTP and 2'-F-UTP) during *in vitro* transcription step to generate 2'-F-modified RNA.

Materials and methods

Recombinant BoNT/A LC

Recombinant BoNT/A LC was produced and purified as described previously [9]. The enzyme concentration was determined using extinction coefficient of $0.83 \text{ (mg/mL)}^{-1} \text{ cm}^{-1}$ at 280 nm [9]. 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 and 2'-F-RNA library

The ssDNA random library consists of a 47 nucleotide random sequence domain which is flanked by constant 5'- and 3'- ends for amplification reactions (5'-GGGAGGAGGAGAGATGTGAACTT-(N47)-AGAACTCTACTGGACTGGCG-3'). The ssDNAs were synthesized by Metabion GmbH (Munich, Germany). Construction of 2'-F-RNA library was detailed in supplementary material section.

SELEX-Process

SELEX was performed on an automated SELEX workstation (highly modified BMK 2000 roboter, Beckman Coulter) similar as previously described [10]. The modifications are listed in the supplementary material section.

Binding analyses of enriched libraries

Binding analyses of enriched libraries was performed with nitrocellulose filter retention assays. Briefly, α -³³P-GTP (Perkin Elmer) labelled RNA of selection cycles 1, 6 and 9 (~80nM) was incubated with increasing concentrations of LCA (12.5nM–500nM) in a final volume of 25 μ l binding buffer (PBS pH7.4, 3mM MgCl₂, 5mM DTT 1mg/ml BSA, 1mg/ml heparin) and was subsequently filtered on a dot blot manifold (Minifold, Whatman). The fraction of nucleic acid co-retained in a complex with the target protein on the nitrocellulose membrane (Protran 0,45 μ m, Whatman) was washed five times with 200 μ l of washing buffer (Phosphate Buffer Saline (PBS) pH7.4, 3mM MgCl₂, 5mM DTT, 0.05% Tween) and subsequently quantified with a phosphorimager (Fuji FLA 5000).

Cloning and sequencing

The PCR product of cycle 9 was cloned (TOPO TA Cloning Kit, Invitrogen) according to the manufacturer's instruction. Twenty one colonies were subsequently picked and grown overnight in 5 ml of LB medium, and the plasmid DNA from these colonies were harvested using the Wizard SV96 Plasmid DNA Purification System (Promega). Plasmids with monoclonal inserts were sequenced using the MWG Eurofins Operon sequencing facility (Ebersberg, Germany). Different monoclonal sequences were amplified by PCR from their respective plasmids, and in vitro transcribed into α -³³P-GTP labeled RNA as described in supplementary material section.

Binding analyses of monoclonal sequences

Binding analyses was performed with nitrocellulose filter retention assay as described above with the difference that it was performed in a higher stringency binding buffer (PBS pH7.4, 300mM NaCl, 3mM MgCl₂, 5mM DTT 1mg/ml BSA, 0,2% Tween, 1mg/ml heparin) and washing was performed five times with 200 μ l PBS pH7.4, 300mM NaCl, 3mM MgCl₂, 5mM DTT, 0.2% Tween.

Binding Specificity of the aptamers

Specificity testing was performed with the nitrocellulose filter retention assay. About 80 nM of α -³³P-GTP labelled aptamer was incubated with LCA (250 nM) or control proteins (250 nM) (control proteins are listed in the results section), and subsequently filtered through nitrocellulose filter membrane on a dot blot manifold. The membrane was washed with PBS buffer (pH 7.4) with total 300 mM NaCl, 3 mM MgCl₂, 5 mM DTT, 1 mg/mL BSA, 0.2% Tween, and 1 mg/mL heparin. The fraction of nucleic acid-co-retained with protein on the membrane was quantified with a PhosphorImager (Fuji FLA 5000).

Dissociation constant (K_D) determination

K_D determination was performed on nitrocellulose filter retention assay with ~0.8nM α -³³P-GTP labelled aptamers S132B-C11, S132B-C12 and S132B-C22. Binding and washing buffer are as described for monoclonal aptamer binding analyses. Values are mean values from duplicate experiments. Values (% retention) were normalized for the maximum retention level (= fraction bound). Analyses were performed with Kaleidagraph software package.

Inhibition effects of aptamers on the endopeptidase activity of LC of BoNT/A

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). [11]. The detailed procedures of the assay has been previously published [11] and is listed in the supplementary materials section.

Enzyme kinetics

Enzyme kinetics measurements were carried out using the same procedure as outlined above and previously published [11]. Substrate concentrations from 5 to 25 μM were used (e.g., 5, 10, 20, and 25 μM) for enzyme activity with 25 nM of LCA. The detail procedures are listed in the supplementary materials section. Inhibition constants were calculated using Lineweaver-Burk plots derived from experiments conducted in the presence of inhibitor [11].

Analysis of structures of aptamers

Three-dimensional structures of RNA aptamers were predicted using the iFoldRNA algorithm (<http://troll.med.unc.edu/ifoldrna/>, [12]). Structures output was chosen as PDB format. Maximum Entropy based Docking (MEDock) was used for molecular docking between aptamer and LCA (<http://medock.csie.ntu.edu.tw>, [13,14]). No LCA monomer has been crystallized. To obtain structural coordinates of LCA, Protein Data Bank code 3DDA [15] was used. 3DDA is the co-crystal between LCA and its substrate peptide (QRATKM). LCA was manually separated from the substrate peptide. Similar procedures have been used in the literatures to obtain the monomeric LCA [16]. To compare aptamer docking relative to positive control, we used the following formula as the docking fitness:

$$1 - [(A - P) / (P)]$$

where A is the docking energy between aptamer and LCA, P is the docking energy between peptide substrate and LCA. BoNT/A holotoxin (3BTA) was used as a negative binding control [17]. The docking fitness would be 1 for the docking between LCA and its peptide substrate.

Results and Discussions

In vitro selection and sequence analysis of LCA binding aptamers

Sequencing revealed 14 different sequences: 7 sequences were identical (S132B-C12), 1 sequence was identified twice, all other sequences were orphans (including S132B-C11 and S132B-C22). The binding analysis was carried out for these sequences in order to evaluate their binding affinity to the target. As shown in Figure 1, S132B-C11, S132B-C12, and S132B-C22 showed substantial binding to LCA, but not to the His-tag-Sec 7 or lysozyme. These three sequences were therefore chosen for further characterizations. The sequences of the three aptamers are listed in Table 1.

Binding specificity and affinity of aptamers

To investigate the specificity of the aptamers, we choose His-Sec7, His-ERK2, streptavidin, and lysozyme as the control proteins. His-Sec7 and His-ERK2 were chosen because our target protein, LCA has the His-tag; streptavidin was chosen due to the streptavidin-beads used for capture our target protein; lysozyme was used as the non-specific control. From Figure 2a, the three aptamers identified from our in vitro selection showed specific binding to our target protein, LCA. The aptamer clones S132B-C12 and S132B-C22 have better selectivity than S132B-C11.

To investigate the binding affinity, the filter retention assay was carried out. As shown in Table 2 and Figure 2B, all three aptamers exhibit an affinity for LCA in the low nM range, with S132B-C22 showing the strongest binding (87 nM), followed by S132B-C12 (111 nM), and S132B-C11 (186 nM) (Table 2). The binding data demonstrate that the three aptamers possess both high binding affinity and specificity for LCA.

Inhibition activity of aptamers against endopeptidase activity of LC of BoNT/A

The potential endopeptidase inhibitory behaviour of each of the aptamers was also investigated. As shown in Figure 3A, all three aptamers exhibited a dose-dependent inhibition effect against the endopeptidase activity of LA. The IC_{50} values of all three aptamers are in the low to mid nanomolar range (Table 3), with 66 nM for C12. Since the enzyme concentration we used in the inhibition assay is 25 nM, this means that 50% LCA inhibition is achieved when the inhibitor:LCA ratio is 2.6:1 for S132B-C12, and 12.2:1 for S132B-C11 (Table 3). As a control, the inhibition effect of initial library of RNA on the endopeptidase activity was tested. At concentrations as high as 22 μ M the initial library did not possess inhibitory activity. These data demonstrate that inhibition is not due to non-specific interactions between LCA and RNA molecules.

Currently, a limited number of small molecule inhibitors of LCA have been identified [11, 16,18–22], with the IC_{50} only in the low micromolar range. Our results showed that all three aptamers identified here not only have strong binding affinity with LCA, but also exhibit strong inhibition activity against LCA. While most recently, there are reports of selection of DNA aptamers against LCA [23,24], inhibitory activity has not been shown. Our report here is the first report to obtain 2'F pyrimidine stabilized RNA aptamers with strong inhibition activity against the endopeptidase activity of BoNT/A light chain (in nM range).

Enzyme Kinetics

To understand the mechanism of inhibition, we carried out enzyme kinetic studies on all three aptamers. In the absence of the aptamer, the K_M is 23.0 μ M ($\pm$ 0.4 μ M), which is similar to our previously reported value [11]. All three aptamers depict a non-competitive inhibition mechanism from the Lineweaver-Burk plot (Figure 3-B1–B3), with different intercepts at Y axis (yet not being parallel) compared to that in the absence of the aptamer. The inhibition constants for three aptamers are listed in Table 3. The non-competitive inhibition indicates that the inhibitor's enzyme binding site may be different from the substrate:enzyme binding site, and it can interfere both enzyme active site and enzyme-substrate complex. As shown in Table 3, while the K_I of all three aptamers is in around 100 nM range, the K_I 's showed larger differences. K_I ' of S132B-C11 is 163 nM, which is close to its K_I , suggesting the inhibition by S132B-C11 is in a classic non-competitive mode. The K_I ' of S132B-C12 and S132B-C22 (525 and 603 nM respectively), on the other hand, is much greater than their respective K_I . This means that there are higher competitive components in the inhibition by S132B-C12 and S132B-C22.

Molecular modeling

Currently, no molecular modeling has been done on aptamer's three dimensional structure. The published data on aptamers are all based on the secondary structures predicted by MFold [25]. Most recently, Ding et al developed a computational algorithm (iFoldRNA) to predict the three dimensional structure of RNA [12]. We applied this algorithm to predict our RNA aptamers' 3D structures (Fig S-1). When the length of RNA is over 50 nucleotides, the iFoldRNA algorithm requires significantly longer time, and may reduce the prediction accuracy. The aptamers identified from this study consist of 91 or 93 nucleotides (with a constant 3'- and 5'- flanking region and a 47 nucleotide central region). To compare the prediction results, we applied iFoldRNA to both the central region and the full length aptamers.

The predicted structures of the central region alone are similar to those of the full length aptamers (data not shown), suggesting 1) the prediction results for longer aptamers are as good as the shorter ones in our case; and 2) in our case, the flanking regions do not interfere with the folding of the core sequences.

To investigate the nature of the interaction of aptamers and LCA, we explored molecular docking. MEDock is an algorithm based on Maximum Entropy docking for study of protein-ligand docking and interaction [13,14]. MEDock has been applied to small molecule-protein docking, as well as large biomolecule-protein docking (DNA-protein docking). In this report, we applied MEDock to study the docking between aptamer and protein. Since binding and inhibition studies were carried out using the full length aptamers, full length aptamer structures predicted with iFoldRNA were also used for the docking study. The docking results are listed in Figure 4. To validate the MEDock results, we use the LCA-SNAP-25 (197–202) co-crystal (PDB #: 3DDA, [15]) as our positive control. We manually separated the molecular coordinates of substrate peptide (QRATKM) from 3DDA, and input the molecular coordinates from each of the two parts independently (LCA and C-terminus of SNAP-25 (197–202)) to MEDock for docking simulation.

The docking results are very close to the original structure of 3DDA. Surface plasmon resonance binding studies reveal that the aptamers do not bind to the native toxin (unpublished data), thus we have chosen the native toxin (PDB #: 3BTA) as a negative control. Our docking results showed no docking between 3BTA and each of the three aptamers, while the docking fitness percentage (calculated by docking energy) between the aptamers and LCA (relative to the positive control, which is 100%) is 90% for S132B-C22, and 67% and 61% for S132B-C12 and S132B-C11 respectively.

The docking results showed that our aptamers are binding to the surface of the light chain of BoNT/A (Fig. 4). All three aptamers have residues near the active site (zinc) of light chain. For S132B-C11, Cytidine-63 and Cytidine-66 (both are within the central region) are next to the Zn atom. The oxygen atom located on the second carbon of cytidine-63 is closest with a distance of 5.05 Å (Fig. 4A). In addition, the 3'-flanking region (purple color) blocks the entrance of the catalytic center of LCA. Docking results for the S132B-C12 aptamer (Fig. 4B) suggest that portions of the molecule downstream of the core sequence and upstream of the 3' flanking region together form a belt (5'-C₆₇AAGA₇₁-3') which blocks the entrance of the catalytic center and Zn binding site. The nitrogen atom located on the NH₂ group of Guanine (residue 70) is 5.06 Å away from Zn atom. The nitrogen atom of NH₂ group of Adenine (residue 71) is 4.5 Å away from Zn. These two atoms might play a role in the enzymatic inhibition of the function of C12 aptamer. For S132B-C22 aptamer, the closest residue to the Zn atom is Adenine (residue 44), located on the central region, which is 8.16 Å away from Zn (Fig. 4C). The 5' flanking region (dark blue in Fig. 4C) is also close Zn catalytic center. This segment does not directly hinder access to the Zn catalytic center, but rather binding to the LC adjacent to the active pocket. The overall docking results suggested that all three aptamers are close to and interact with the Zn catalytic center, which might play a pivotal role for the inhibition function of the aptamers. Furthermore, after docking with aptamers, the template cannot dock to the substrate peptide (QRATKM), suggesting the competitive nature between aptamers and substrate peptide. All three aptamers also showed the stereo hindrance around alpha-exosite of LCA [26], which could prevent the effective anchoring of the substrate to LCA (see supplementary Fig. S-2). The overall binding to the surface of LCA explains that they might interact with the enzyme-substrate complex, and thus supporting our non-competitive inhibition from enzyme kinetic analysis.

Conclusions

In this report, we successfully identified three RNA aptamers selectively against LCA. These three 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 low nM range. While aptamer S132B-C11 showed a classic non-competitive inhibition with K_I similar to K_I' , aptamer S132B-C12 and S132B-C22 showed more competitive component ($K_I < K_I'$). For the first time, we explored the prediction of the 3D structures of RNA aptamers, and explored molecular docking of the aptamers to their target. Our molecular docking results support our kinetic study of the inhibition of aptamers on the enzymatic activity of LCA. This is the first report to confirm functional aptamers against BoNT/A light chain which will provide the basis to develop these aptamers as therapeutics against the deadly botulism. Besides the potential therapeutic applications of these aptamers, they can also be used for rapid detection of the BoNT and have the potential for the diagnosis for botulism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Binding screenings of monoclonal sequences from RNA pool with the target (light chain of BoNT/A) and control proteins.

A **B** **Figure 2.**

Binding specificity and affinity of aptamers. Panel A, Binding specificity of aptamers to light chain of BoNT/A. Panel B: Binding curve for the aptamers to light chain of BoNT/A. The error bars represent the standard deviation of duplicate measurements.

Figure 3.

Inhibition of aptamers on the endopeptidase activity of BoNT/A LC. Panel A: the dose-response curve for aptamer-mediated BoNT/A LC endopeptidase inhibition. The curve was fit using non-linear polynomial regression. Panel B: Lineweaver-Burk plot of BoNT/A LC inhibition with and without inhibitor aptamers (B-1, aptamer S132B-C22; B-2, aptamer S132B-C12; B-3, S132B-aptamer C11). The error bars represent the standard deviation of six measurements.

Figure 4.

RNA aptamer docking with light chain of BoNT/A. The red ball on the light chain represents zinc. Blue ribbons represent the 5'-flank region, the purple ribbons represent the 3'-flank region, the cyan ribbons represent the core sequence of the aptamer. Panel A, S132B-C11; panel B, S132B-C12; and panel C, S132B-C22. Detail interactions between aptamer and light chain are described in the text part of the article.

Table 1

Core Sequence of aptamers

S132B-C22	GACAGCGUGCCUAGAAGUCCAAGCUAAAUAACCACGCUCGACAAGC
S132B-C12	ACAACCCGGAACAACGUCUAAACAGUGUACCAUAACCCGGCAUUA
S132B-C11	AUUCGGGCCCAGGAACCAACUAUAUAAAUGUCCCGAAUGCUUCGACG

Table 2

Binding affinity (Dissociation Constants) of aptamers to LCA

	Dissociation Constant (K_D) (nM)
S132B-C22	87 (20) *
S132B-C12	111 (21)
S132B-C11	186 (18)

* Data in parenthesis are the standard deviation of the duplicate measurements

Table 3IC₅₀ 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)
S132B-C22	214 (9) *	8.6	124 (17)	603 (75)
S132B-C12	66 (2)	2.6	106 (24)	525 (40)
S132B-C11	304 (13)	12.2	108 (15)	163 (18)

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