Strategies to Design Inhibitors of Clostridium Botulinum Neurotoxins

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Abstract: Botulinum neurotoxins (BoNTs), produced by spore-forming anaerobic *Clostridium botulinum*, are the most toxic substances known. They cause the life-threatening disease botulism, characterized by flaccid muscle paralysis. While the natural cases of botulism are rare, due to their extreme toxicity and easy production, BoNTs have become potential biowarfare agents, and create maximum fear among populations concerned with bioterror agents. The only available antidote against BoNTs is equine antitoxin. Equine antitoxin can only target the toxins at extracellular level, and can not reverse the paralysis caused by botulism. In addition, equine antibody can cause severe hypersensitivity reactions, and is limited to be used for prophylaxis treatment.

BoNTs are large proteins with three distinct domains, the binding domain, the translocation domain, and the enzymatic domain with highly specific endopeptidase activity to cleave the proteins involved the neurotransmitter release. Targeting any of these domains can inhibit the functions of BoNT. Humanized monoclonal antibodies, small peptides and peptide mimetics, receptor mimics, and small molecules targeting the endopeptidase activity have emerged as potential new inhibitors against BoNTs. With the structure of BoNT resolved, molecular modeling and rational design of potent antidotes against botulism is on the horizon. An area that has not been explored for designing the antidotes against botulism is aptamers, which have been successfully developed as therapeutics in several areas. This review will focus on some of these new strategies to design effective antidotes against botulism. The strategies reviewed in this article can be easily applied to design inhibitors for other bacterial toxins.

Keywords: Botulinum neurotoxin, inhibitor, therapeutics, prophylactics, antibody, aptamer.

INTRODUCTION

Clostridial botulinum neurotoxins are the most toxic substances presently known [1]. Botulinum neurotoxins (BoNTs) are a group of proteins produced by different strains of *Clostridium botulinum,* and are responsible for botulism disease. Seven different stereotypes, designated as A to G, are known to exist. BoNTs are produced as a ~150 kDa single polypeptide chain. The protein is post-translationally proteolyzed to form a dichain in which the heavy chain (HC, ~100 kDa) and light chain (LC, ~50 kDa) are linked through a disulfide bond [2, 3]. HC is composed of two 50 kDa domains, with the N-terminal half involved in translocation and the C-terminal half involved in binding with nerve cells. LC is confined to catalysis only [2, 4] (**Fig. (1)**). According to the x-ray crystal structure, BoNT/A and /B have a similar sequential arrangements of the three functional domains. The three functional domains are structurally distinct except for a loop which wraps around the perimeter of the catalytic domain (**Fig. (2)**; [5, 6]).

Botulism is a severe disease characterized by flaccid muscle paralysis, which is caused by BoNT-mediated blockage of acetylcholine release at the nerve-muscle junctions. A combination of features such as neuronal target site, efficient cellular entry, and unique enzymatic activity lends BoNTs extreme toxicity, with a mouse lethal dose of 0.1 ng/kg [7]. Although all serotypes of BoNT share similar structure and ultimate function of the blockage of the neurotransmitter release, epidemiologically only types A, B, E and F are known to cause human botulism [8].

While naturally occurring botulism cases are rare, BoNTs create maximum fear among population as bioterror agents due to their high toxicity and relatively easy production [9]. Contamination of restaurant, catered or commercial foodstuffs, or beverages could cause illness in large number of consumers [10]. Aerosol exposure of BoNTs does not occur naturally, but could be attempted by bioterrorists to achieve widespread effect. A single gram of crystalline toxin, evenly dispersed and inhaled, would kill more than one million people, although technical factors would make such dissemination difficult [8]. A more realistic scenario suggests that less than one gram of BoNT, if distributed into food supply, such as milk, could cause more than 100,000 casualties [9]. BoNTs are one of the six most dangerous biothreat agents (Category A agents) listed in National Institute of Allergy and Infectious Diseases (NIAID) and Centers for Disease Control and Prevention (CDC) (http://www3.niaid.nih.gov/Biodefense/bandc_priority.htm), and are the only toxin group in the category A list. The challenge of developing more effective treatment for botulism has been recognized by NIAID, and has been among NIAID's highest priorities [11]. The detailed structural study of BoNT and understanding of the mechanism of the action of BoNT in recent years have provided basics for the new strategies in designing new generation of antidotes against BoNT.

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MODE OF ACTION OF BOTULINUM NEUROTOXINS

As part of their mode of action, BoNT and tetanus neurotoxin (TeNT) interact with presynaptic nerve cells. The steps involved in the cell intoxication can be divided as follows [12]: binding, internalization, membrane translocation and inhibition of neurotransmitter release (**Fig. (3)**). Neurotoxin binds to a receptor on the cell surface through the C-terminal domain of HC (Hc), and is internalized via receptor mediated endocytosis. The N-terminal domain of HC (H_N) is involved in mediating translocation of the LC into cytosol of the neuronal cell presumably through a membrane channel induced by the low pH of endosomes. The interchain disulfide bond is then reduced and the LC is translocated and released into the cytosol. Inside the cytosol, the LC inhibits neurotransmitter release by destroying exocytotic docking/fusion machinery. LC acts as a Zn^{2+}-endopeptidase against specific intracellular protein targets present either on the plasma membrane or on the synaptic vesicle.

Receptor Binding

It is shown that BoNT binds to the presynaptical membranes of the nerve muscle junctions. The cellular receptor of BoNT is currently subject of intense research, but its nature is still unclear. A model that takes into account all the experimental findings suggests that BoNT binds through a double-receptor system consisting of a protein receptor and acidic lipid-gangliosides through its Hc domain [13].

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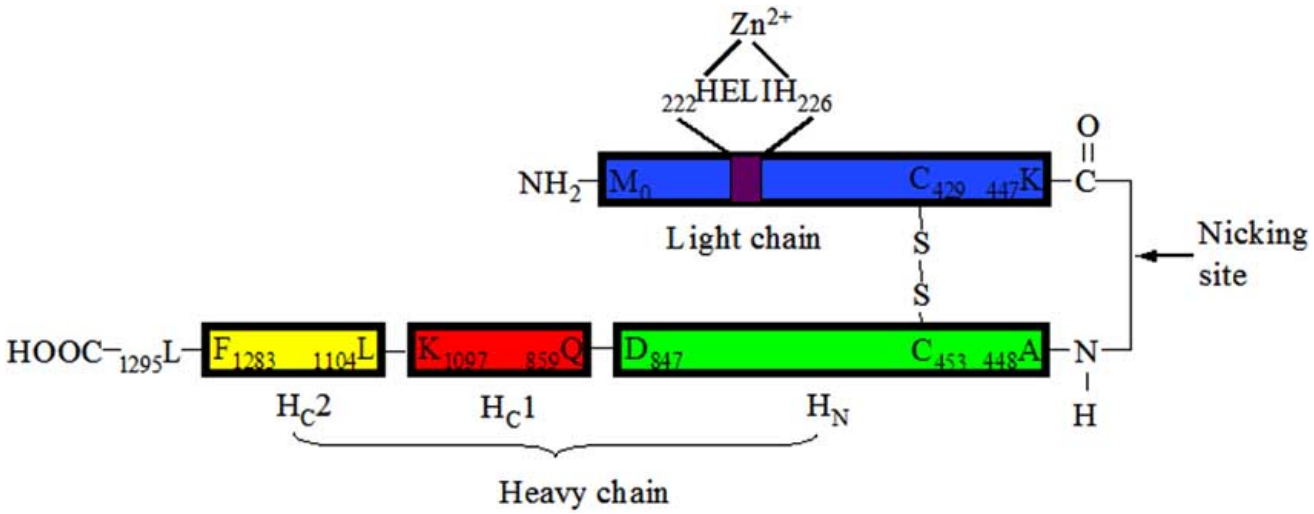

Fig. (1). Schematic diagram of BoNT/A showing different domains.

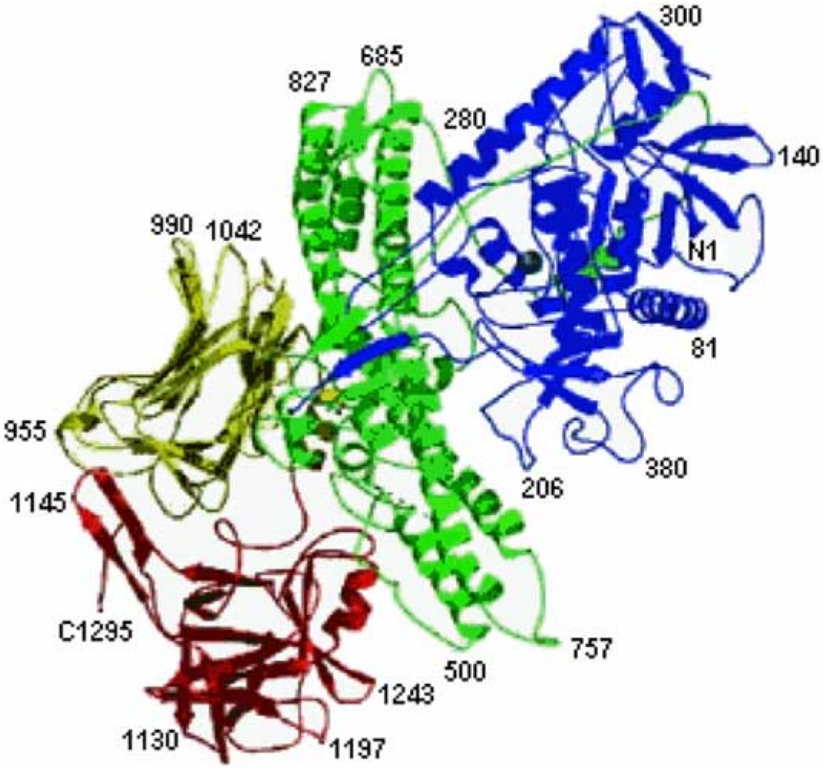

Fig. (2). Three-dimensional representation of the backbone BoNT/A [5]. The catalytic domain (LC) is colored in blue, translocation domain (H_N) in green, the N-terminal and C-terminal domains of H_C in yellow and red, respectively. The catalytic zinc is depicted as a ball in gray.

GT_{1b} gangliosides are thought to act as a first receptor for the BoNTs [14, 15]. Since gangliosides are dispersed on the neuronal surface [16, 17], they are present in very large excess compared to BoNT, and are expected to diffuse laterally as fast as the other membrane lipids. Thus, gangliosides would form an effective system to capture the neurotoxin and deliver to its protein receptor [13].

Ganglioside binding, which is a low affinity site, is suggested to alter the BoNT structure to make it compatible for binding with the protein receptor [13, 17]. Synaptotagmin I and II have been identified as the receptor initially for BoNT/B [18, 19], and more recently for BoNT/A [20], /E [20], and /G [21]. Efforts have also been made to identify other protein receptors for BoNT. Adherence of botulinum and tetanus neurotoxins to synaptosomal proteins of bovine brain was studied by Schengrund *et al*. [22]. Both toxins appeared to adhere preferentially to an ~80 kDa and to a lesser extent to an ~116 kDa protein. Both proteins were found to be glycosylated. Blasi *et al*. [23] reported identification of a glycoprotein of approximately 140,000 MW from the presynaptic plasma membrane of cholinergic nerve terminals from *terpedo* electric organ, as a possible BoNT/A receptor. Very recently, SV2, a synaptic vesicle protein, has been identified as the receptor for BoNT/A [74].

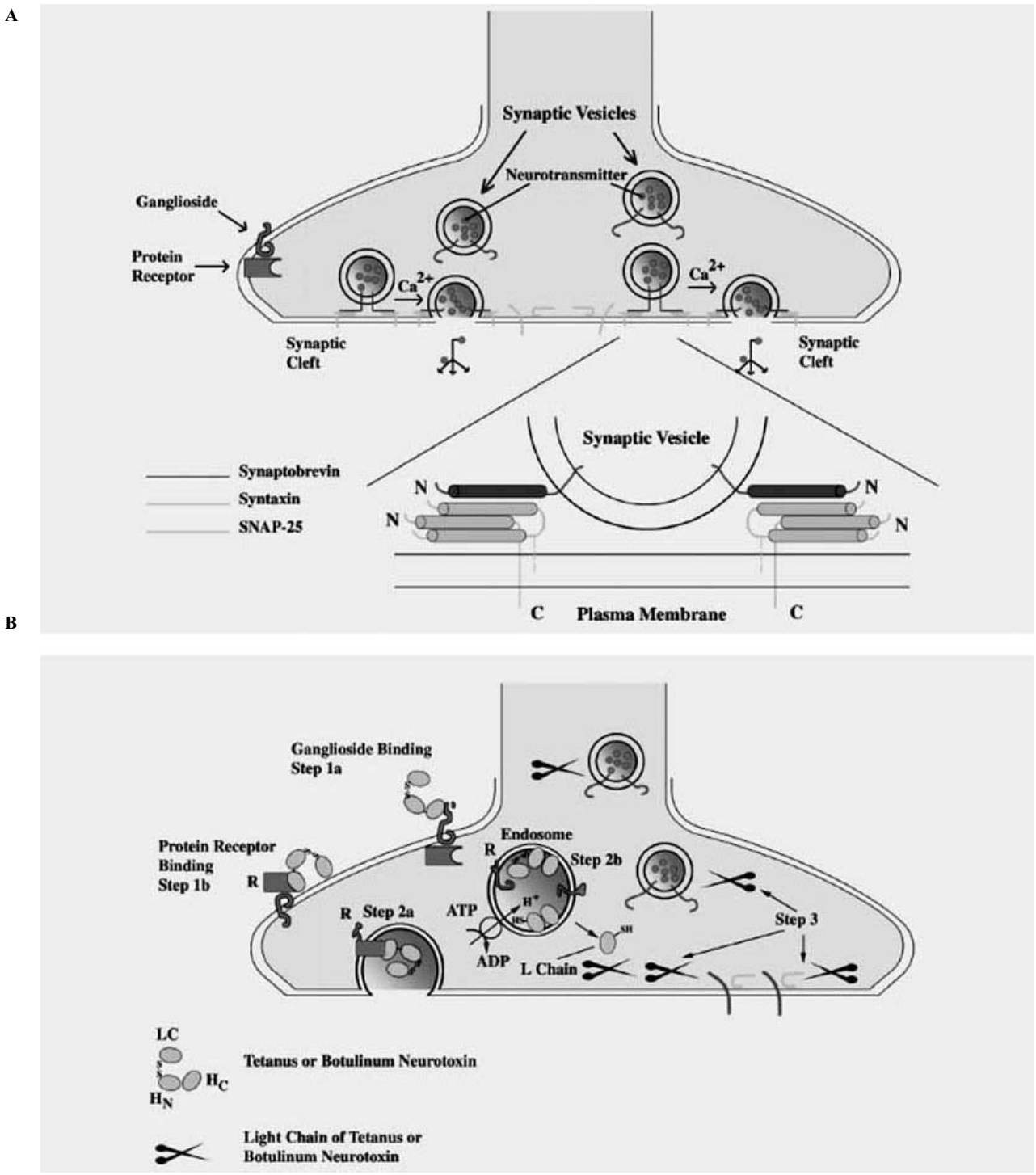

Fig. (3). Schematic models of the neurotransmitter release and the actions of botulinum and tetanus toxins (Derived from [1]).
a, Synaptic vesicles containing neurotransmitters dock with plasma membrane through SNARE proteins (synaptobrevin, syntaxin and SNAP-25). Neurotransmitters are released through a Ca^{2+}-triggered fusion process. SNARE proteins remain in random coil conformations until associated in the SNARE complex at docking, where they form a helical bundle. b, Botulinum or tetanus toxin binds to the presynaptic membrane through gangliosides and a protein receptor (step 1); it is internalized through endocytosis (step 2a), and its L chain is translocated across the membrane (step 2b). The L chain acts as specific endopeptidase against either synaptobrevin (on synaptic vesicles), syntaxin (on the plasma membrane), or SNAP-25 (on the plasma membrane). BoNTs (or TeNT) cleave their substrates before the SNARE complex is formed.

Internalization and Translocation

After cell surface binding and receptor-mediated endocytosis of the neurotoxin, an acid-induced conformational change in the neurotoxin's translocation domain is believed to allow the translocation domain to penetrate the endosome to form a pore. The membrane interaction and pore formation is thought to facilitate the passage of at least the catalytic domain across the membrane into the cytosol [2, 25]. The details of how the translocation domain changes conformation at acidic pH to form a pore and how it can allow for the passage of a 50 kDa catalytic domain across the endosomal membrane are the least understood aspects of the botulinum intoxication mechanism. Membrane channel activity by recording ion transport and fluorescent dye release, and also the catalytic domain (LC), have been commonly used to examine the translocation step of botulinum neurotoxins [25-27].

Intracellular Catalytic Action

The final mechanistic step in toxicity is the cleavage of a presynaptic protein by the BoNT endopeptidase activity. Neurotoxins block the neurotransmitter release by cleaving the vesicle associated membrane protein (VAMP, also called synaptobrevin), syntaxin, or synaptosome associated protein of 25 kDa (SNAP-25) (**Fig. (3)**). It is known that the L chain subunit of the neurotoxin, once inside the nerve cells, is sufficient to block the neurotransmitter release [28-32]. Botulinum and tetanus neurotoxins are zinc-proteases, and the substrates for different serotypes of BoNT and TeNT are the constitutive components of the secretory machinery [2, 12, 24, 33]. The intracellular substrates of L chains of different types of BoNTs vary, and are components of the synaptic vesicle docking and fusion complex: VAMP, the substrate for BoNT/B, BoNT/D, BoNT/F and TeNT; synaptosomal associated protein of 25 kDa (SNAP-25), the substrate for BoNT/A, BoNT/E and BoNT/C1; and Hpc-1/syntaxin, the substrate for BoNT/C1 [34-37]. These substrates are present not only in nerve cells but also in certain other secretory cells, such as PC 12 cells [38], chromaffin cells [39] and pancreatic endocrine cells [40]. The uniqueness of the BoNT is their substrates are highly specific for each serotype, even when they share the same substrate (VAMP for BoNT/B, /D, and F; SNAP-25 for BoNT/A, /E, and C), the cleavage site is distinct for each serotype [3]. The molecular basis of this site-governing substrate specificity of different serotypes of BoNT is not clearly understood. A secondary recognition site beyond the cleavage site of SNARE proteins, which apparently interacts with the active site and/or an allosteric site of BoNT L chain, has been proposed to understand this unique feature [41]. A conserved SNARE motif in SNARE proteins has been identified to serve as recognition sequence for binding of SNARE proteins with BoNT L chains (**Fig. (4)**, [41]).

It has been shown that BoNTs also cleave relatively short synthetic peptide substrates. For example, the segment containing residues 187-203 of mouse brain SNAP-25 is a substrate for BoNT/A [42], while the peptide comprising residues 167–186 of SNAP25 is a substrate for BoNT/E [43]. The segment containing residues 60-94 of human VAMP-2 is substrate for BoNT/B [44], and the VAMP-2 segment containing residues 37-75 can act as a substrate for BoNT/D and F [45].

CURRENT STATUS OF THERAPEUTICS AGAINST BOTULISM

The flaccid muscle paralysis caused by BoNTs cant last for several months [46]. Patients who have already developed the syndrome have to be put under respiratory intensive care for weeks to months [8, 10, 47]. The estimated cost for each botulism patient under respiratory supportive care could be as high as $350,000 [9]. This puts a large burden on hospitals, both financially, and infrastructurally.

Equine antitoxin therapy (neutralizing antibodies against BoNT/A, /B, and /E; and an investigational heptavalent (against ABCDEFG) antitoxin) is the only available antidotes against botulism. BabyBIG, an IgG preparation derived from the blood of volunteers vaccinated with pentavalent botulinum toxoid (ABCDE) vaccine has been developed specifically to treat infant botulism, and is available only on a limited basis. Antitoxins given within 24 hour of the onset of disease can lower the death rate of the botulism and shorten the duration of the symptom [10, 47]. While antidotes like equine antitoxin are essential and effective in prophylactic treatment, there are known pitfalls. First of all, the antitoxin administration is temporal. It has to be administrated at right time, preferably before or shortly after the exposure to be effective. Delaying the administration of the antitoxin eliminates the effectiveness of the antitoxin, since the antitoxins are effective only in binding and neutralizing BoNT that has not yet been endocytosed by peripheral cholinergic neuronal cells, and it can not get into the nerve cell to neutralize the toxin. Also, equine antitoxin can lead to

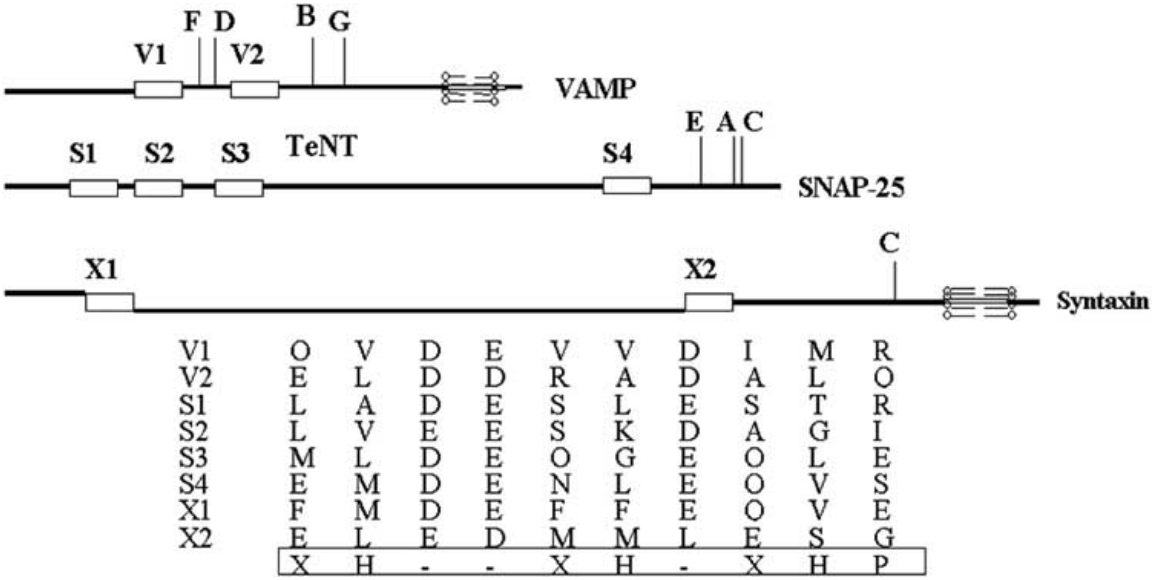

Fig. (4). Positions of SNARE motif in three substrate responsible for the target specificity of clostridial neurotoxins [41]. The motif consists nine residues which is common to all three substrates: hydrophobic residue (h), Asp or Glu residue (-), polar residue (p), and any residue (x).

severe adverse effects in patients, such as uticaria, serum sickness, and anaphylaxis [8, 10, 47]. The prophylactic treatment requires an extreme safety profile. In times of a bioterror attack, many people will not know their exposure status, and a safe prophylactic treatment is critical for the post-exposure population. The adverse effects of equine antitoxin have limited its use as a prophylactic treatment for botulism, and equine antitoxin is not an effective antidote due to the fact it does not reverse existent paralysis.

Significantly, there is no efficient method available for early diagnosis of botulism. The standard mouse bioassay takes four days to complete and can not meet the clinical need for diagnosis. Therefore, the diagnosis of botulism relies on the syndrome development (12-72 hour after exposure [8, 10]), and is confirmed by mouse bioassay. The syndrome of botulism is often confused with other neuromuscular disorders, such as Guillian-Barré syndrome, myasthenia gravis, or stroke [8, 10], therefore, making an early diagnosis of botulism more difficult. Without effective early diagnosis, the therapy for botulism is largely supportive, including artificial ventilation under the intensive care.

There is an investigational pentavalent (ABCDE) toxoid available for vaccination of high risk population (such as lab workers working on BoNTs and military personnel to prevent possible attack). Many new generations of vaccine against BoNT are under development, and have been reviewed by Byrne and Smith [52], therefore, those will not be reviewed in this article. However, an important issue regarding the vaccination against BoNT need to be mentioned. The mass immunization against botulism is not feasible and desirable, because the botulism outbreak is rare (except for the intentional exposure in a potential bioterror attack). More importantly, BoNT is an effective therapeutic agent against numerous neuromuscular disorders, and a wide range of cosmetic applications, as well as many other clinical applications currently under human clinical trials.

NEW STRATEGIES TO DESIGN INHIBITORS AGAINST BONT

With the detailed mode of action of BoNT understood, many new strategies can be used to develop more potent inhibitors and antidotes against BoNT. Therapeutics against BoNT can target any of the three steps of mode of action of BoNT: binding, endocytosis/translocation, and endopeptidase activity, as well as neutralization of toxins in the extracellular milieu. While targeting exctracellular neutralization and binding of BoNT to cell surface will provide effective prophylactic treatment and prevention measures to botulism, the therapeutic treatment to reverse the paralysis syndrome will require an effective and specific delivery system to deliver the therapeutic agents that have the ability to effectively block the endopeptidase activity of BoNT into the damaged nerve cells.

ANTIBODY

Currently, passive antibody administration is the only available medical countermeasure for botulism, along with supportive therapy. The antitoxin available is from the equine anti sera immunized with the BoNT toxoid (equine antitoxin) and the human botulinum immune globulin derived from volunteers vaccinated with the investigational pantavalent botulinum toxoid (BabyBig®) for infant botulism. The resulting polyclonal antibodies (Abs) consist of hundreds to thousands of different Abs which bind to many different parts of the toxin and can neutralize toxins with high potency. However, polyclonal Abs, such as equine antitoxin and BabyBig®, involve hyperimmunizing horse or human volunteers, and plasmapheresis, making a large scale manufacturing inconvenient.

In addition, the side effects related to equine toxin (the only approved therapy for adults), such as serum sickness and hypersensitivity reactions, make it difficult to be used as an effective prophylaxis for post-exposure population. Recent study showed that at equimolar concentrations of equine immunoglobulin G and toxin protein, the biological activity of toxin can be enhanced [48]. This observation suggests that a conformational change of toxin may occur upon binding to the certain epitopes, and enhances the toxicity, and indicates that the adequate amount of antibodies is needed for the therapy. In addition, the polyclonal antibodies derived from human or animal sources have the possibility of transmission of infectious agents.

On the other hand, neutralizing monoclonal antibodies (mAbs) against botulinum have been generated [49-51, 53]. These mAbs have the potential therapeutic applications [49]. MAbs can be manufactured in unlimited supply, do not require a source of immune donors, are consistent batch to batch, and have no infectious risk. Humanized technology can yield an Ab with 90% or higher in human sequence, and far less immnunogenic in humans than murine mAbs. More recently, it has been proven that it is possible to make fully humanized mAbs by immunizing mice transgenic for the human immunoglobulin locus (XenoMouse technology) and making the hybridomas [54, 55]. The humanized mAbs can also be generated using phage display technology by cloning the repertories of human Ab genes into the bacterial phage vector where the Abs are displayed on the surface of the bacteriophage fused to one of the phage coat proteins [56]. Humanized mAbs provide a new avenue for treating botulism with less side effects. The fact that mAbs recognize only a single epitope means that they have limited neutralization power against patho-gens that exhibit antigenic variation. While the epitope which the mAb recognized is important, it has been reported that the specific epitope is less important in neutralizing the BoNT (it does not mean the epitope is not important) [49]. So far, no single mAb can significantly neutralize BoNT [49, 53]. This problem can be circumvented by generating mAb cocktails, and it has been shown that a combination of three mAbs can neutralize 450,000 times of 50% lethal doses of BoNT/A [53]. Such preparations, however, would likely encounter a more complex regulatory process. In addition, the treatment window is short for antibody-based therapy, since they can only neutralize toxins at the circulation level. By combining specific delivery system, those mAbs (or the fraction of mAbs) can be delivered into the BoNT intoxicated nerve cells (see the delivery system section), and working at the intracellular level.

SMALL MOLECULES

Peptide Based Inhibitor

Based on the substrate information, several small peptides have been developed as competitive inhibitors for the BoNT endopeptidase activity. Based on the cleavage site sequence of the BoNT/A (E A N **Q R** A T, Q and R is the cleavage site for BoNT/A), short peptides have been designed to achieve high inhibition effect on BoNT/A endopeptodase activity ([45], Table **1**). Short peptides containing a common sequence of CRATKML have been identified as the effective inhibitors of BoNT/A. Further modification of this common sequence by replacing cysteine with 2-mercapto-3-phenylpropionyl has yielded a peptide with Ki of 330 nM [58].

In another approach, phage display technology has been used to design small peptide inhibitors against BoNT. Phage display is a powerful tool that extends the range of modern combinatorial screening techniques, allowing the discovery and characterization of proteins that interact with a desired target [59]. Screening small peptide library using the phage display technology has yielded several promising small peptide-based inhibitors targeting the endopeptidase activity of BoNT [60]. Most of the identified peptides have the complete inhibition effect on the BoNT in a millimolar to micromolar concentration range. At least, these

Table 1. Short Peptide Inhibitors of BoNT Endopeptidase Activity [45]

Peptide	Sequence*								Inhibition constant K_i (mM)
			P_2	P_1	P_1'	P_2'			
1	C	Q	R	A	T	K	M	L	0.19
2	d-C	Q	R	A	T	K	M	L	0.14
3		C	R	A	T	K	M	L	0.0019
4		d-C	R	A	T	K	M	L	0.0018
5	N	C	R	A	T	K	M	L	0.5
6	A	C	R	A	T	K	M	L	0.15
7	N	d-C	R	A	T	K	M	L	0.15
8	A	d-C	R	A	T	K	M	L	0.026
9	C	d-C	R	A	T	K	M	L	0.11
10		C	d-R	A	T	K	M	L	0.41

* P1-P1' is the scissile-bond of BoNT/A.

peptides can be used as lead compounds for designing more potent inhibitors.

Hinge peptide mini libraries containing the acidic amino acids Asp and Glu (conseevetive in the SNARE motif, **Fig. (4)**), the scissile-bond amino acids Gln and Arg (for BoNT/A), and the zinc chelators His and Cys has been constructed [61]. Each library has the structure of acetyl-X1-X2-linker-X3-X4-NH2, or X1-X2-linker-X3-NH2, where X1, X2, X3, and X4 are one of the six amino acid residues in the hinge library, and the flexible linker is 4-aminobutyric acid. The libraries have shown pronounced inhibition on BoNT/A protease activity. Deconvolution of these libraries is likely to provide new leads for design of the BoNT inhibitors.

Similar strategies have also been used for screening the inhibitors on endopeptidase activity of BoNT/B and TeNT. BoNT/B and TeNT share the same substrate (synaptobrevin) and the same cleavage site (Gln^{76}-Phe^{77}). The pseudopeptides mimicking the sequences that may interact with the synaptobrevin sequence Gln^{76}-Phe^{77} have been designed as potential inhibitors against BoNT/B and TeNT (**Fig. (5)**, [62, 62]). A tripeptide surrogate has been designed based on subsites of the catalytic domain that are putatively interacting with the cleavage site of synaptobrevin (S_1-S_1'-S_2', where S_1 the left of the cleavage bond and S_1' and S_2' at the right). Using β-amino thiol derivatives to replace the side chain at the S_1 has led to strong inhibitor against the endopeptidase activity of BoNT/B with a Ki value of 20 nM [63].

Targeting SNARE motifs provide another alternative to design a protease inhibitor against BoNTs [64]. As mentioned earlier, a unique feature of BoNTs has been their exclusive substrates and cleavage sites [3]. The SNARE motifs have been thought to play important role in the secondary recognition between LC and its specific substrate. Targeting these recognition sites may prevent the productive binding of BoNT to the SNARE proteins, and block the proteolytic action of BoNT on their substrates. One version of this repeating motif in VAMP (synaptobrevin), designated V2 with the sequence of ^{62}ELDDRADALQ^{71} (**Fig. (4)**), can block the neurotoxic actions of both BoNT/A and /B when the peptide is injected into cultured Aplysia neurons [41, 64]. While the therapeutic agents targeting the active site will have to be tailored to individual variants of BoNT, agents based on the SNARE motif have the potential to treat poisoning by all forms of BoNT as well as tetanus.

Peptide based drugs have two major limitations, poor bio-availability, and short duration of action. Therefore, the peptide mimetics should be designed based on the pharmacophoric groups responsible for the agonist activity. The crystal structures of BoNT have been resolved [5, 6, 65-68]. The 3D structures provide critical information about the molecular mechanism of BoNT action, as well as information on how to design the inhibitors against them. Molecular docking and modeling between the lead peptides and BoNT can lead to identify of the pharmacophoric groups for designing the effective peptidomimetics as inhibitors against BoNT.

SMALL MOLECULE LIBRARY SCREEN

Many drug-like small molecule libraries are available now commercially as well as in national repositories. Screening these drug-like compounds has become critical in finding new therapeutic candidates. Screening such libraries requires a robust assay feasible for the high throughput screening. Such assays have been developed for screening the endopeptidase activity of BoNT [45, 69, Ambrin *et al*, 2005[1]], making it feasible to find inhibitors against the protease activity of BoNT by screening large library of compounds. By screening diversified small molecule (non-peptidic) libraries, potential inhibitors can be found for further confirmation and rational design to improve the potency. This strategy has been used for screening inhibitors against BoNT [70, 71]. A common pharmacopore has been identified from this approach (**Fig. (6)**, [70]). This provides scaffolds for designing more potent inhibitors against BoNT.

The assays used for high throughput screening is important for successful screening. Currently the high throughput screening used for endopeptidase activity of BoNT is based on short synthetic peptides as substrates, and based on the fluorescence as the signal readout [45, 69, Ambrin *et al*, 2005[1]]. The data from these screenings need to be independently confirmed by the secondary assay, because short peptides are not the optimized substrates for BoNT, and the library compounds may interfere with the fluorescence based assay. The confirmatory assays are needed with full

[1]Data have been presented at the second annual retreat of NERCE. Ambrin G.; Cai, S. and Singh, B. R. (2005) Differential endopeptidase activity of different molecular forms of type A botulinum neurotoxin, New England Regional Center of Excellence/Biodefense and Emerging Infectious Diseases Second Annual Retreat, September 25-26, 2005, Durham, NH.

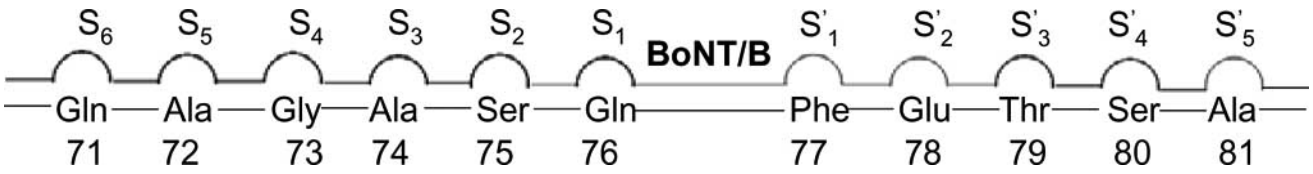

Fig. (5). Human synaptobrevin II sequence surrounding the scissible bond (^{76}Gln-Phe^{77}). The side chains of the different synaptobrevin residues are putatively interacting with subsites of the catalytic domain of BoNT/B designated S1 to S6 on the left of the cleavage bond and S1' to S5' on the right ([62, 63]).

length protein substrates. The screenings carried out so far are using light chain of BoNT as the surrogate [45, 69]. This may pose another possible question on whether the light chain by itself behaves identical to BoNT intracellularly? It has been known that active state of BoNT is in a more dynamic state than that available from the crystal structure [72, 73]. While the crystal structure of LC itself is similar to that in the holotoxin, it is possible that there are differences when they are in solution. Therefore, using the entire toxin for screening may be more appropriate.

Another obstacle for the endopeptidase inhibitor is how to deliver the molecules into the intoxicated nerve cells. We will describe this issue in a later section of this review.

RECEPTOR MIMICS

Another clinically relevant target to design antagonists against botulism is to block the binding between BoNT and its receptors. It has been proposed that the binding of BoNT to nerve cells is through a two-step process, first by binding to the gangliosides, followed by the high affinity binding to a protein receptor(s) [13, 17]. Synaptotagmin has been proposed as the protein receptor for BoNT/A, /B, /E, and /G [18-21]. More recently, the synaptic vesicle protein SV2 has been identified as the receptor for BoNT/A [74]. Those proposed receptors for BoNT provide a pool of targets to design receptor mimics as the antagonists to block the binding between BoNT and the nerve cells. For example, a portion of synaptotagmin II plus ganglioside can effectively block BoNT/B action in mice [79].

We have cloned the receptor binding domain of BoNT/A (C-terminus quarter of heavy chain of BoNT/A, or HCQ) with the functional binding activity to gangliosides and the synaptotagmin [75]. We have demonstrated that the quinic acid can inhibit the binding between HCQ and the ganglioside at the concentration of 10 mM (**Fig. (7)**, Cai *et al*, 2005[2]). The sugar mimics may lead to stronger inhibition of binding between BoNT and the gangliosides. In addition, we have found a peptide derived from synaptotagmin to show protection against BoNT/A challenged in a mouse bioassay (Cai *et al*, 2005[2]).

While receptor mimics are valid targets for designing inhibitors against the botulism, like antibody based therapy, the treatment window for this class of agents is short, since they can only target at the circulation level. Once the toxin gets into the nerve cells, effectiveness of receptor-based inhibitors will be very limited.

APTAMERS

Aptamers are unique oligonucleotides that have high affinity for their targets (proteins or the small molecules). Technically, aptamers could be isolated against any protein known to mankind [76-78]. Aptamers form unique structures that provide basis for high affinity and specificity towards their targets. Thanks to specific and tight interactions, aptamers serve as valuable tools to modulate or block functions of proteins. The screening process for aptamers is popularly termed as SELEX (Systematic Evolution of Ligands through EXponential enrichment) [77]. The process of SELEX starts with generation of a library of randomized DNA or RNA sequences (**Fig. (8)**). Usually this library contains 10^{14} to 10^{15} different DNA or RNA species that fold into different structures, depending on their particular sequence. The library is incubated with the target protein of interest, and those DNAs or RNAs present

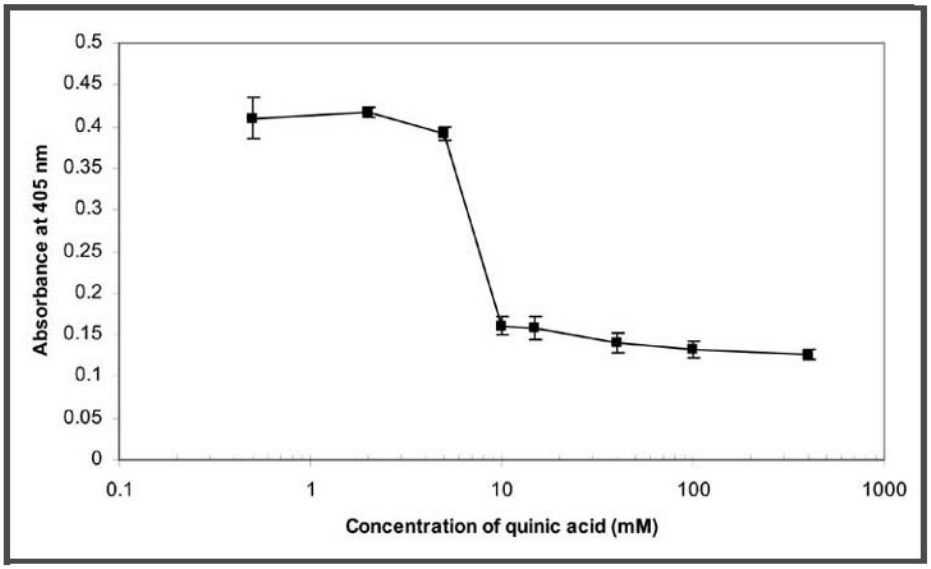

Fig. (7). Competitive inhibition binding assay of qunic acid on the binding between GT1b and HCQ (Cai *et al*, 2006[2]).

[2]Data have been presented at the 2006 annual RCE Meeting. Cai, S.; Singh, B. R.; Sharma, S. K.; Sharma, S.; Zhou, Y. (2006) Novel design of inhibitors against botulinum neurotoxin targeting its receptors, 2006 Annual Regional Centers of Excellence Meeting, New York, NY, 2006.

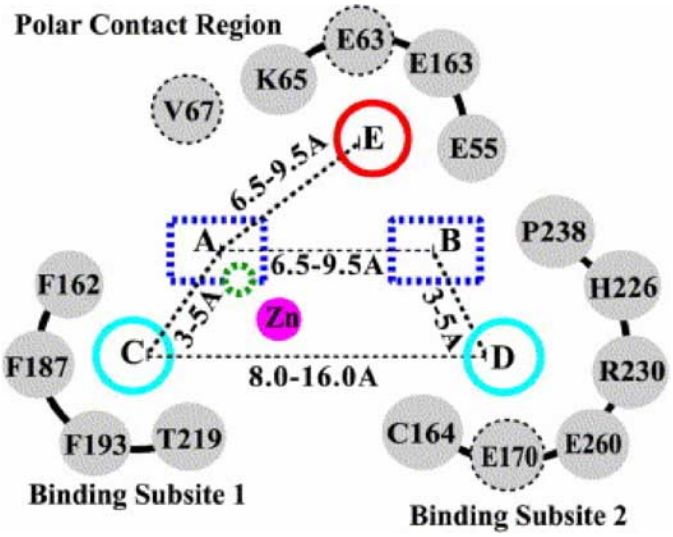

Fig. (6). A pharmacophore for BoNT/A LC inhibition [70, 71]. Planar components A and B are blue dashed rectangles, one of which contains a heteroatom that may engage in an interaction with the enzyme's catalytic zinc, or potentially replace the water used by the zinc engine during substrate lysis. Hydrophobic components C and D (light blue circles) are predicted to interact with subsites 1 and 2 in the substrate binding cleft, respectively. The positive ionizable component of the pharmacophore (E) (the red circle) is hypothesized to either engage in electrostatic or water mediated interactions with residues in the polar contact region. Gray spheres are residues that remained consistent when docking inhibitors in predicted binding subsites of both dynamics and molecular mechanics 'only' refined models. Residues E63, V67, and E170 (the gray spheres with dashed black boarders) are found to participate when docking inhibitors in dynamics structures [71].

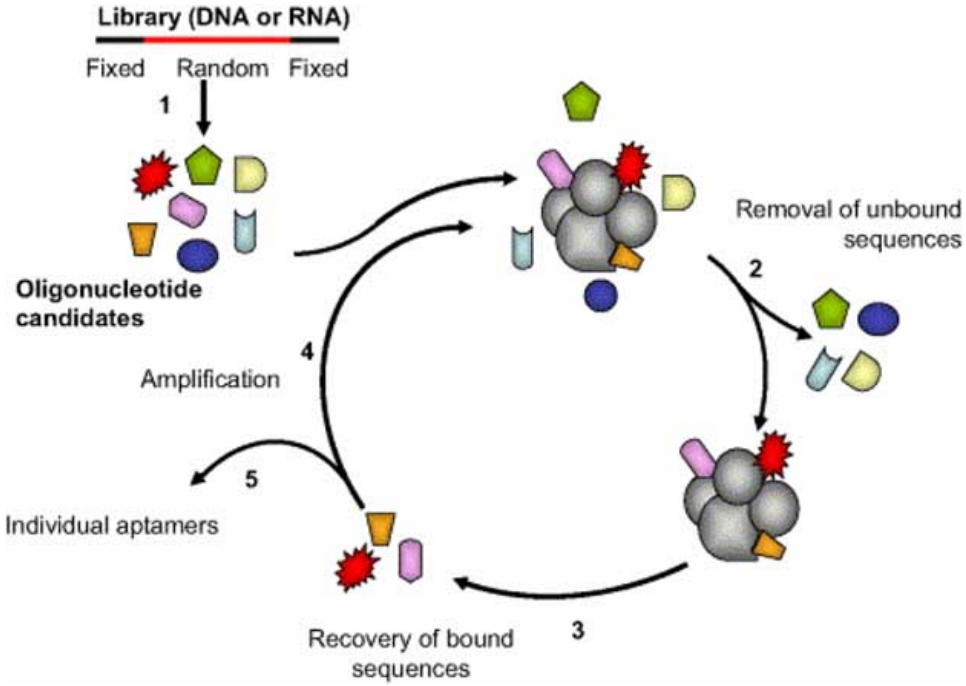

Fig. (8). Scheme of SELEX. A library of DNA or RNA molecules is incubated with the protein target, and those that bind to it are separated from the rest. The bound sequences will be amplified by PCR (RT/PCR in case of a RNA library). The selected pool then enters a new round of selection. During these iterative rounds of selection, the population evolves towards the sequences with strongest binding affinity with the protein target [76].

in the library that bind to the protein are separated from those that do not. The retained DNAs or RNAs are then amplified by PCR (in case of RNAs, by reverse transcription/PCR and *in vitro* transcription) to generate a pool of DNAs or RNAs that have been enriched for those that bind to the target of interest. This selection and amplification process is repeated (usually 4 to 20 rounds) until the winning DNA or RNA ligands with the highest affinity for the target protein are isolated. The winning ligands are defined as aptamers that are then cloned and sequenced.

Several properties of aptamers that make them as attractive therapeutic and diagnostic agents rival, and in some cases, even surpass antibodies. Those characteristics are listed in Table **2**. Binding of an aptamer is a highly specific interaction, with its strong ability to discriminate targets on the basis of subtle structural differences [80, 81]. The properties of aptamers make them ideal for both therapeutic and diagnostic applications.

Aptamers rival antibodies in terms of affinity and specificity for targets (Table **2**), and are considered very promising for *in vivo*

Table 2. Aptamers vs. Antibodies

Aptamers	Antibodies
Binding affinity high (in low picomolar to low nanomolar, in term of dissociation constant, K_d)	Binding affinity high (in low picomolar to low nanomolar, in term of dissociation constant, K_d)
Entire selection is an *in vitro* chemical process, and therefore can target any proteins, small molecules, and whole organisms	Selection needs a biological system, and therefore difficult to raise antibodies against toxins or non-immunogenic targets
Iterative rounds against the targets limits screening processes	Screening monoclonal antibodies time consuming and expensive
Uniform activity between batch to batch	Activity of antibodies vary from batch to batch
Wide variety of chemical modifications to molecule for diverse functions	Limited modifications of molecules
Return to original conformation after temperature insult or other chemical denaturation process	Temperature and environment sensitive and undergo irreversible denaturation
Unlimited shelf life	Limited shelf life
No reported immunogenicity	Significant immunogenicity
Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse the effect of antibodies
Can select for ligands under different conditions for *in vitro* diagnostics	Limited to physiological conditions for optimizing antibodies for diagnostics
Easy to label reporter molecules (such as fluorescence dye) to the aptamers at precise location identified by user	Difficult to label the reporter molecules at precise location

therapy. Aptamers are widely applied towards a variety of targets, from small molecules to proteins, and even for whole organisms [82, 83]. Most *in vivo* targets for aptamer based therapy are at extracellular levels, which are easier to access, and represent less stringent pharmacological challenge. Aptamers have been used for several areas as investigational therapies. They have been used to target coagulation factors as anticoagulants [84, 85]; the vascular endothelial growth factor (VEGF) for array of diseases, such as cancers, retinopathies, and age-related macular degeneration [86-88]; virus proteins for HIV, influenza and hepatitis C [89-91]; antibodies and cytokines for autoimmune diseases and inflammation diseases [92-95]; neuropathological targets for neurodegeneration diseases, such as Alzheimer's disease and transmissible spongiform encephalopathies (TSEs) [96-100]; membrane biomarkers for cancer [101, 102]. The first aptamer based drug, Macugen®, has been approved by FDA for age-related macular degeneration [88].

Aptamers as a class have demonstrated little or no toxicity or immunogenicity [103]. During the chronic toxicity study on rats and woodchucks with high levels of aptamer (10 mg/kg daily for 90 days), no toxicity is observed by clinical, cellular, or biomedical measures [104]. AS1411, an oligonucleotide aptamer that binds to nucleolin, a protein expressed on the surface of tumor cells, is currently in the extended phase I study. In a dose-escalating phase I trial in patients with various advanced cancers, no serious adverse events have been observed up to a dose of 22 mg/kg/day during single continuous 7-day iv infusion (corresponding to a mean peak plasma concentration of 1.5 μM), and the observation of additional cases of stable disease in renal cell carcinoma (RCC) supports the efficacy of anti-cancer effects in this disease [105]. These results support both efficacy and safety of aptamer based drugs during systematic delivery.

Due to small size (about 10 kDa, compared to 150 kD antibodies), aptamers are likely to produce better tissue penetration. Also, aptamers are chemically synthesized at relatively low cost, and have better batch-to-batch reproducibility. Additionally, aptamers can be modified to adjust the pharmacokinetic profile. Further, to reverse the effect produced by aptamers, antisense molecules against aptamers could be used [85].

Unmodified aptamers have a short half-life in the blood circulation due to the rapid nuclease degradation, and the fast clearance rate. This is an advantage for some therapeutic areas, such as thrombin aptamer for anticoagulation therapy, but is likely to be a problem for the majority of other *in vivo* applications, including botulism therapy. Chemical modifications have been developed to improve the nuclease resistance of oligonucleotides [98, 106]. When conjugated to high-molecular-weight polyethylene glycol (PEG), the half-life of aptamers is much longer (9-12 h) [107]. Aptamers also can be formulated into a long-lasting formulation by making emulsion with the biodegradable polymer microsphere (such as poly-lactide-glycolide (PLG)) [108], which can achieve the half-life of months. The tunability of the pharmacokinetics makes aptamers as good candidates for many therapeutic areas.

While aptamers are easier to access the extracellular targets, when combined with the appropriate delivery system, they can also target the intracellular targets. Technically, delivery of aptamers will be similar to delivery of any nucleic acid based compounds such as antisense oligonucleotides and siRNAs. Special formulations (carriers) are increasingly becoming available to transport nucleic-acid based drugs to all parts of body, including across the blood-brain barrier [109-113]. Extensive work is underway and numerous approaches are under development to achieve this formidable task [109, 110].

The advantages of aptamers make them as great candidates for antidotes against botulism. Aptamers can be developed as the neutralizing agents targeting the toxins at the extracellular level, with added advantages over antibody-based therapeutics (see the discussion above). Aptamers can target at all three functional domains of BoNT. Aptamers not only can be used as the prophylactic treatment for the post exposure population, but also can be used as the therapeutic treatment. Aptamers targeting the endopeptidase activity of BoNT can be used as the therapeutic agents to reverse the clinic syndrome of botulism when combined with a proper delivery system.

Because of the relatively easy screening process, development of aptamer-based therapy is comparatively fast. It takes as less as 36 months from selecting target to bringing it into the clinical trial

[114]. This is particularly important for development of the therapeutics against bioterror agents like BoNT. While currently, there is little reported research on using apatmers as therapeutic agents for BoNT (and on the bioterror agents, in general), we believe aptamer-based drugs are very good candidates for bioterror agents (including BoNT).

DELIVERY SYSTEM

The inhibitors targeting the protease activity of BoNT have the potential to reverse the clinical effects of intoxication, the paralysis. However, this requires a targeted delivery system which can specifically deliver the inhibitors into the intoxicated nerve cells. The natural biology and ability of the BoNT heavy chain to be endocytosed and translocated may represent an opportunity to use it as a delivery vehicle for inhibitors. Heavy chain (HC) of the BoNT has been shown to be an effective delivery vehicle to deliver molecules into the BoNT specific nerve cells [115, 116]. BoNT HC itself is not toxic, and therefore, provides a safe delivery vehicle. The inhibitors can directly be conjugated with HC, or can be encapsulated with HC anchored liposomes. Inhibitors to be delivered by HC can be small molecule inhibitors against the endopeptidase activity of BoNT, antibodies (or the fraction of antibodies) inhibiting the protease activity of BoNT, or the aptamers targeting the protease activity of BoNT. One drawback of this approach is that anti-heavy chain antibodies may be induced, thus preventing future medical use of BoNTs in individuals using this delivery system for botulism treatment.

CONCLUDING REMARKS

Like other infectious disorders, the detailed mechanism of BoNT pathogenicity and the structure-function relationship of BoNT provide invaluable targets for development of the antidotes and inhibitors against botulism. The BoNT molecule is divided in clear functional domains that can operate independently. This feature provides multiple targets for designing therapeutics to treat botulism. High throughput screening and the combinatorial chemistry provide another useful tool for screening the inhibitors against botulism. Combining the random screening (such as the phase display, small molecule library screening, SELEX) and rational design will greatly facilitate the drug discovery process and improve the potency and bioavailability of the potential drugs for treatment of botulism.

Another important area need to be developed is to establish the valid animal model to test the *in vivo* efficacy of the therapeutic agents against BoNT. It is difficult to design human clinical trial for testing the efficacy of any potential drugs for botulism, therefore, the efficacy data are mostly likely to come from animal model (especially in primates).

While development of the treatment for botulism is a high priority in times of bioterror threat, due to the rareness of botulism, there are not enough incentives for large pharmaceutical companies to invest for development of such therapies. It is likely to heavily rely on academic institutes, government agencies, and small biotech companies to develop such drugs. It is estimated that development of each new drug costs about $1 billion dollar [117]. How to more effectively coordinate such efforts becomes another critical point for successful development of the therapeutic agents against botulism. While this is beyond the scope of this review, we think that is an important issue which needs to be addressed, not only for development of effective treatment for botulism, but also for development of therapeutics against other biothreat agents, concerned with only small patient populations.

ACKNOWLEDGEMENT

This work was in part supported by a DoD/Army Contract No. W911NF-06-1-0095 and by the National Institutes of Health through the New England Center of Excellence for Biodefense Grant AI057159-01). We also thank Ms. Sapna Sharma and Dr. Vesa Nevalainen for sharing their unpublished data on ganglioside-based antidotes against botulism.

ABBREVIATIONS

BoNT	=	Botulinum neurotoxin
TeNT	=	Tetanus neurotoxin
HC	=	Heavy chain
LC	=	Light chain
HCQ	=	C-terminus quarter of heavy chain of BoNT/A
mAb	=	Monoclonal antibody
SELEX	=	Systematic evolution of ligands through exponential enrichment
NIAID	=	National Institute of Allergy and Infectious Diseases
CDC	=	Centers for Disease Control and Prevention
SNAP-25	=	Synaptosome associated protein of 25 kDa
VAMP	=	Vesicle associated membrane protein

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Received: July 4, 2006 Accepted: November 10, 2006