EXPERT OPINION

Current strategies for designing antidotes against botulinum neurotoxins

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Introduction: Botulinum neurotoxins (BoNTs) are proteins responsible for the deadly paralytic disease botulism. Extreme toxicity, ease of production and lack of antidotes against BoNT makes it a category A biothreat agent, according to the United States Center of Disease Control and Prevention. The only available therapy for BoNT is an equine antitoxin antibody or/and a protracted respiratory support system. Even then, antibody treatment can only prevent further exposure of the toxin and cannot rescue already intoxicated neurons.
Areas covered: In this article, the authors provide a summary of the current status of inhibitors and antitoxins used against BoNTs. In particular, the authors focus on new strategies used in the development of novel therapeutics. They also outline the major steps involved in BoNT's mechanism of action and identify specific inhibitors for each step.
Expert opinion: Several previous efforts have resulted in less than satisfactory results that are due, in part, to a lack of sustained effort in addition to a poor understanding of the unique structural features of the toxin. BoNT is a double-edged sword with both toxic effects and therapeutic benefits, excluding vaccination as a preventative measure. The long lasting intracellular endopeptidase activity, which causes an extended period of muscle paralysis, necessitates the need to identify effective inhibitor(s) against BoNT, and this could ultimately lead to new therapeutic options.

Keywords: biothreat, botulinum, clostridium, drugs, endopeptidase, inhibitors, neurotoxin, therapeutics

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1. Introduction

The deadly disease botulism is caused by seven distinct serotypes (A – G) botulinum neurotoxins (BoNTs) produced by various strains of *Clostridium botulinum*, an anaerobic Gram-positive bacterium listed as 'Category A' biothreat agent by the United States Center of Disease Control and Prevention and poses a great threat to public health. The bacteria are ubiquitously found in soil and water. BoNTs are the most toxic substances known to mankind with a mouse LD_{50} of 0.1 ng/kg for the type A [1-3].

Four clinical forms of botulism have been recognized: food borne, infant, wound and inadvertent. Food-borne botulism is the most common, which occurs from the oral ingestion of improperly preserved foods, such as ham, sausages, fish and vegetables, which provide conditions for the growth of *C. botulinum*, whereas in the US, infant botulism is the most common form of botulism.

Currently, there is no effective antidote available against botulism except for the equine antibodies to remove BoNTs in blood circulation. After exposure to the BoNT, the only effective lifesaving treatment is mechanical ventilation. However, ventilation is an impractical option in the case of large-scale exposure, such as a terrorist attack. There is an urgent need to develop both prophylactic and therapeutic

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Article highlights.

- Botulinum neurotoxins being the most toxic substances known to mankind pose serious threat to public health, and are also being used as therapeutic agents against numerous neuromuscular disorders, present a unique challenges in countermeasures. Vaccination of the general population is not a desirable option, given the therapeutic use of the toxins.
- Antitoxin antibodies that are currently being used and developed have limited window for only removing circulating toxins in patient's system.
- Several peptidic and non-peptidic small molecule candidates have been identified as potential inhibitors for targeting binding, translocation and intracellular action of botulinum neurotoxins.
- Inhibitors against the endopeptidase activity, the most critical action of the toxin intracellularly, have been developed as leads, but further developments have not been successfully carried out so far.
- Sustained efforts are needed with multidisciplinary teams to develop drugs against botulism.

This box summarizes key points contained in the article.

agents against BoNT for preventing and treating botulism disease. The challenge of developing a more effective treatment for botulism has been recognized by National Institute of Allergy and Infectious Diseases (NIAID) and has been among NIAID's highest priorities, but progress has been slow.

2. Structure and mechanism of action of BoNT

BoNTs are produced as a 150 kDa single polypeptide chain in the cytosol of C. *botulinum* and are released into the culture medium after bacterial lysis. The 150 kDa toxin produced in the bacteria is in the form of a complex, containing the neurotoxin and neurotoxin-associated proteins or NAPs [4,5]. The 150 kDa protein is post-translationally proteolyzed (nicked) in certain serotypes by bacterial proteases to form the biologically active dichain neurotoxin, composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC), linked through a disulfide bond and noncovalent protein interactions [6].

The three functional domains of botulinum toxins are the LC and two 50-kDa domains of the HC, the C-terminal of HC (H_C) and the N-terminal of HC (H_N). The molecular mechanism of BoNT action is not well understood. Based on the existing experimental evidence, a three-step working model has been proposed (**Figure 1A and B**) [2,7-10].

2.1 Extracellular step

Neurotoxin binds to a double receptor consisting of gangliosides G_{T1b} and a protein on the nerve cell surface through the H_C. In accordance with this model, BoNT/A receptor binding consists of the synaptic vesicle (SV2) protein and acidic lipid polysialogangliosides, specifically G_{T1b}, G_{D1b} and G_{Q1b} through the H_C. The BoNT binding to the neuronal cell receptors is serotype-specific [11-14], whereby different serotypes of BoNT bind to either synaptotagmin or SV2 protein. More recently, fibroblast growth factor receptor 3 is reported as a high affinity receptor for BoNT/A in neuronal cells [15].

2.2 Internalization and translocation steps

On binding, the neurotoxin is internalized through endocytosis. The H_N is involved in mediating translocation of the LC into cytoplasm of the neuronal cell, presumably through a membrane channel induced by the low pH ~ 5 of endosomes and neutral pH in the cytosol. The redox gradient of pH reduces the disulfide bond between HC and LC. The LC domain passes across the endosomal membrane through HC pores into the cytosol [16] where it can act on the intracellular targets [17]. The unique and highly specific ability of the HC binding makes it an excellent drug delivery vehicle for the neuronal diseases [18]. The translocation process exploits the SV recycling pathway and increases SV recycling leading to increased uptake of the toxin into the neurons. The redox reaction due to pH change can be a target for blocking the BoNT's action.

2.3 Intracellular step

Inside the cytosol, LC acts as a Zn^{2+}-endopeptidase against specific intracellular protein targets, present either on the plasma membrane or on the SV, and inhibits neurotransmitter release by destroying exocytotic docking/fusion machinery. BoNTs catalyze proteolysis of specific proteins of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors complex (SNARE complex), which function in the exocytotic machinery [2,9]. The SNARE complex consists of a four-helix bundle, two helices derived from a 25-kDa synaptosome-associated protein (SNAP-25), one from synaptobrevin-2, and the other from syntaxin. BoNT/A,/C and /E cleave SNAP-25 [19-21], whereas synaptobrevin is the target of BoNT/B,/D,/F,/G and tetanus neurotoxin (TeNT) [22-26]. In addition to SNAP-25, BoNT/C also cleaves syntaxin [20,27]. Even though many serotypes share the same substrate, their cleavage sites are distinct and highly specific making each a unique metalloprotease. Finding inhibitors against this endopeptidase activity will have the real potential to treat botulism, as the blockage of the endopeptidase activity can restore the neurotransmitter release. The high toxicity of BoNT poses a great challenge for development of both diagnostics and appropriate treatment of botulism.

3. Current status of therapeutics against botulism

Current therapy for botulism involves respiratory supportive care and the administration of antitoxins. The only antitoxins available are equine antitoxin (neutralizing antibodies against BoNT/A,/B and/E); and an investigational heptavalent (against serotype ABCDEFG) antitoxin for the adult.

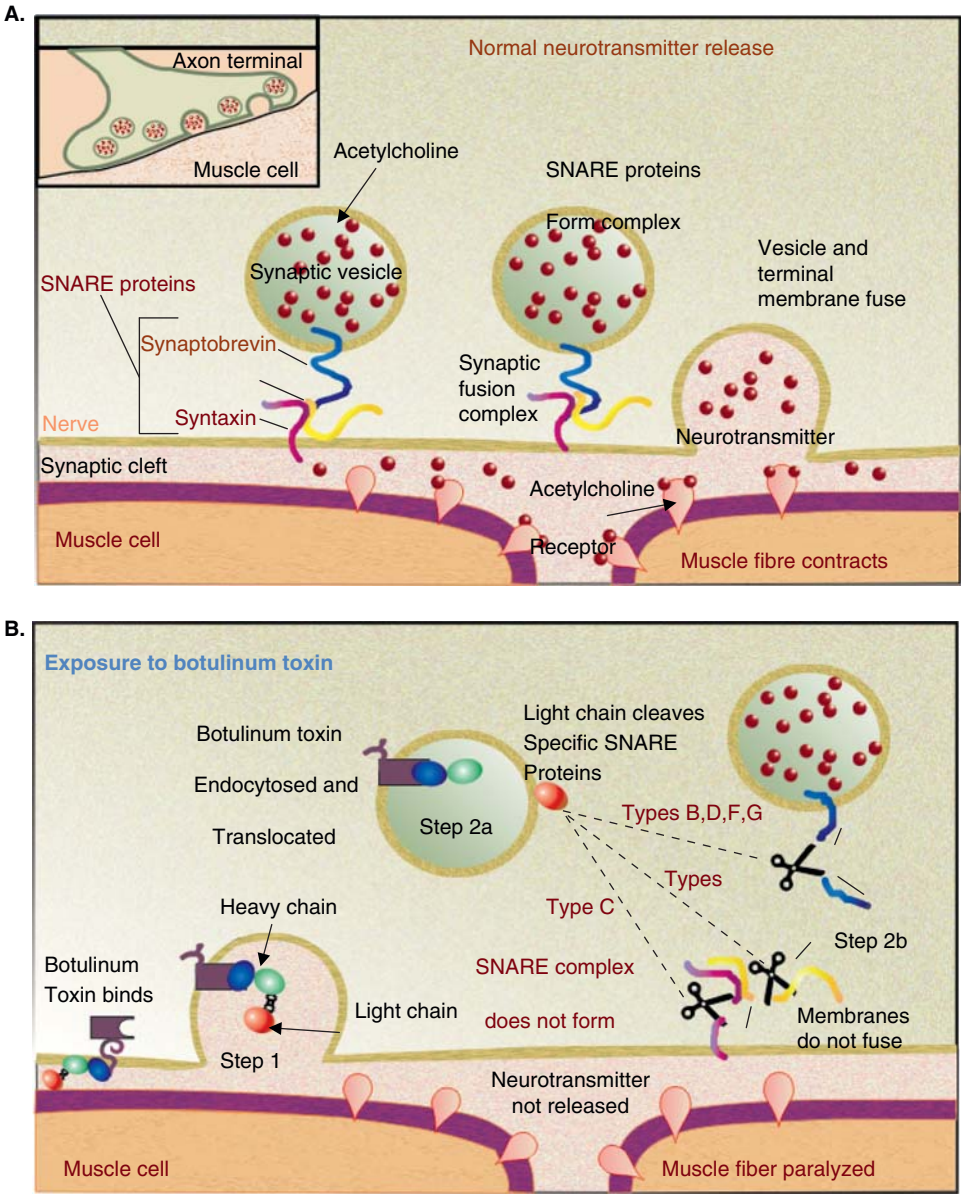

Figure 1. Schematic models of the neurotransmitter release and the actions of botulinum and tetanus toxins [120]. A. Synaptic vesicles containing neurotransmitters dock with plasma membrane through soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors complex (SNARE) proteins (synaptobrevin, syntaxin and 25-kDa synaptosome-associated protein [SNAP-25]). Neurotransmitters are released at the neuromuscular junctions. **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 light chain is translocated across the membrane (step2b). The light chain acts as specific endopeptidase against either synaptobrevin (on synaptic vesicles), syntaxin (on the plasma membrane) or SNAP-25 (on the plasma membrane). Botulinum neurotoxins (or tetanus neurotoxin) cleave their substrates before the SNARE complex is formed.

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To treat infant botulism, BabyBIG®, an IgG preparation derived from the blood of human donors vaccinated with a pentavalent (ABCDE) toxoid vaccine is developed [28]. In addition to removing the circulating toxin, these antitoxins also block further binding of the toxins to the neuronal cells.

It should be noted that the antitoxin has to be administered before toxins can reach the nerve cells. The treatment window for using antitoxin is short; once the syndrome is developed, the antitoxin is not effective, because the antitoxin cannot enter the nerve cell to neutralize the toxin. The flaccid muscle paralysis caused by BoNTs lasts for several months [29].

Patients who have already developed the set of symptoms may have to be put under a mechanical respirator for several months [1,30]. This puts a large burden on hospitals, in both financial and facility resources. In an event of a bioterrorist attack, a public health system may be in crisis due to the lack of effective antidotes against botulism, both for prophylactic and post-exposure treatments. Also, the symptoms associated with botulism are often confused with other neuromuscular disorders, such as Guillain–Barré syndrome, myasthenia gravis or stroke [3,31], thus making an early diagnosis of botulism more difficult. The mass immunization is not feasible or desirable, because a botulism outbreak is rare. More importantly, BoNT is an effective therapeutic agent against numerous neuromuscular disorders and has a wide range of cosmetic applications [32]. Although antibody therapy is currently available and also being further developed for prophylactic treating and neutralizing circulating toxins, even in patients already down with botulism, large-scale antibody production, stockpiling and logistics of their use are cumbersome because of the requirement of government-regulated facilities and handling of large amounts of dangerous toxins. Alternative approaches are needed to develop small drug-like molecules and more robust antidotes for both prophylactic and therapeutic treatments.

With the current knowledge of the mode of BoNT actions, several new strategies can be used in developing potent inhibitors and antidotes against BoNT. BoNT therapeutics can target any of the following discrete steps: binding, endocytosis/translocation, and endopeptidase activity, as well as neutralization of toxins in the extracellular milieu. Targeting extracellular neutralization and binding of BoNT to the cell surface will provide effective prophylactic treatment and preventive measures for botulism. Therapeutic treatment to reverse the paralysis syndrome will require an effective and specific delivery system for the therapeutic agents to effectively block the endopeptidase activity of BoNT into the damaged nerve cells.

Three major approaches are available to develop antidotes against botulism. These are prophylactic antibodies, non-peptidic small-molecule inhibitors and small peptide-based drugs [33]. Recently small RNA aptamers have also been developed as inhibitors for BoNTs [34]. The treatment window is short for antibody-based therapy requiring early detection and diagnosis. Therefore, the two other approaches involving small molecule drugs (peptidic and non-peptidic) are becoming increasingly more important.

3.1 Antibody-based prophylactic therapy

As mentioned earlier, currently available antibody-based therapeutics are equine antitoxin and BabyBIG, both being polyclonal antibodies. Although a passive immunization using these immunoglobulins for prophylaxis is possible, an exceptional safety profile is needed, since many individuals to be treated do not know their actual exposure status.

Within the past decade, efforts have been successfully made to develop neutralizing monoclonal antibodies (mAbs) as viable substitutes for polyclonal antisera, due to their relative ease of production, batch consistency and no infectious risks involved, hence providing newer avenues to treat botulism with lesser side effects. However, no single mAb has been able to significantly neutralize BoNTs [35,36], and a cocktail of mAbs were required, suggesting that effective antibody therapy against BoNT intoxication may require synergistic effect of several antibodies working together [35]. In partnership with NIAID, recombinant mAbs that effectively protect against botulism resulting from all subtypes of BoNT/A,/B and /E are currently being produced and tested for FDA licensure by Xoma Corp., CA, USA. Antibodies that protect against other toxin serotypes (C, D, F and G) are also under development. In the past few years, a 15 – 20 kDa single domain antibody (V_HH) that binds specifically to LC of BoNT serotype A was produced from a humanized-camel VH/V_HH phage display library. The V_HH has high sequence homology (> 80%) to the human VH and could block the enzymatic activity of the BoNT [37].

3.2 Non-peptidic small molecule as an antidote of BoNT

Non-peptidic small-molecule inhibitors, once developed into drugs, have an advantage of higher stability and membrane permeability to reach the target [38,39], in the case of nerve cells poisoned by BoNT. Targeting therapeutics at any of the three steps involved in BoNT mode of action can provide a treatment against botulism.

3.2.1 Non-peptidic inhibitors for the extracellular binding

The development of an inhibitor against the extracellular binding step is the first target of prophylactic treatment. An antagonist against either the cell receptor or the toxin receptor binding domain can be developed. In the past our laboratory has demonstrated that quinic acid can inhibit the binding between HcQ and the ganglioside receptor at a concentration of 10 mM [33]. Another reported inhibitor from natural sources, *Limax flavus* and *Triticum vulgaris*, are lectins [40]. Lectins have affinity for sialic acid at the level of the plasma membrane and serve as a competitive inhibitor for various serotypes of BoNT and tetanus toxin. Similar to the antibody-based therapy, the therapeutic window for this type of antidotes is limited, but such molecules can be effective prophylactically and in combination with drugs against the intracellular endopeptidase activity.

DNA intercalator doxorubicin binds to the neurotoxin serotype B at the receptor-binding H_C domain. The crystal structure has indicated the glycone moiety of the doxorubicin, stacks up with tryptophan 1261 and interacts with histidine 1240 of the binding domain [41]. Although theoretically it is possible to develop drugs against the binding step, given the effort needed to develop drugs against the seven serotypes, each with ganglioside and protein as coreceptors, it is not a

preferred approach for botulism therapy, more so when antibodies could be easily available to remediate the prophylactic situation.

3.2.2 Non-peptidic inhibitors for BoNT translocation and K^+ channel blockers

The acidification of the endocytic vesicles for low pH-mediated translocation of the LC into the cytosol provides another target to develop antidotes against botulism. Ammonium chloride and methylamine hydrochloride antagonize internalization/translocation of toxins by cholinergic nerve endings [42]. Several antimalarial compounds have been screened against the HC of BoNT/A. These compounds delayed BoNT/A-induced muscle paralysis in the mouse hemidiaphragm assay [43,44]. For example, aminoquinolines block the acidification of the endosomes, preventing channel formation by HC for the translocation of LC into cytosol. An endosomal acidification inhibitor, quinacrine, in combination with the BoNT LC Zn metal chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylenediamine, a known Zn chelator of BoNT LC which delays muscle paralysis [45,46], provides better protection against both serotypes A and B than the individual drugs. Chloroquine indirectly affects the BoNT translocation by effectively increasing the endosomal pH [47].

Bafilomycin A1 is another inhibitor that prevents the neurotoxin action by disturbing the redox action potential of the H^+-ATPase pump [48]. Results have suggested that drugs such as bafilomycin A1 protect the muscles from BoNT if applied prior to or during toxin exposure, and this protective effect is diminished when applied after toxin exposure. Toosendanin, a triterpenoid natural product, has also been found to interfere with HC activity or its pore formation in PC12 cells [49]. Further studies from the same group reported that toosendanin could protect monkeys from BoNT/A-, BoNT/B- and BoNT/E-induced death in a dose-dependent manner when coadministered with, or several hours after, neurotoxin administration. Fischer *et al.* have further explored a semisynthetic strategy to identify inhibitors based on toosendanin. Toosendenin acts against BoNT action by arresting the LC translocation through HC channel [50,51]. It should be noted that although toosendanin is generally considered toxic, the doses used for effective protection against BoNT action have been shown to be nontoxic to mice [51].

Similarly, nigericin and monensin, ionophores, at nanomolar concentrations increase membrane permeability to H^+ and K^+ or H^+, Na^+ and K^+, respectively, thus blocking vesicle acidification to neutralize pH gradients. These compounds delay the effects of BoNT/A and BoNT/B up to two to threefold over onset times in unprotected muscles. However, higher concentrations of the ionophores directly blocked synapses. Thus, nigericin and monensin could delay onset of BoNT paralysis only over a narrow range of concentrations [52]. Keller *et al.* have shown pH-dependent translocation blockage by pretreating neuronal cell cultures with concanamycin A (Con A), which prevents cleavage of SNAP-25 with an IC_{50} value of 25 nM. Notably, addition of Con A at times up to 15 min after toxin exposure abrogated BoNT/A action, but addition of Con A after 40 min was no longer protective [53].

There is an entirely different class of compounds, potassium channel blocker/calcium channel activators, which act as antagonists to BoNT. Another reagent that effectively interferes with the translocation of the BoNT is diaminopyridine (DAP). A potassium channel blocker 3,4-DAP has been reported to antagonize BoNT action in a dose-dependent manner on the mouse phrenic nerve hemidiaphragm [54]. As a result of the K^+ channel blockage by 3,4-DAP, action potential is prolonged leading to increased Ca^{2+} influx. The intracellular increase of Ca^{2+} partially overcomes the paralysis caused by the BoNT through increased release of acetylcholine. However, there has been a concern about the safety of DAP use because of the high doses required to block BoNT/A action. Recently, 3,4 DAP in combination with roscovitine (a calcium channel activator) has proven to be more effective inhibitor because the combination reduces the concentration of DAP for the blockage of BoNT/A action [55]. This approach is, however, limited to only BoNT/A, as Ca^{2+}-influx only relieves BoNT/A-mediated blockage of acetylcholine release.

One of the main features of disrupting the membrane translocation of the toxin, or increased Ca^{2+} influx, is by interfering with the membrane channel activity. The inhibitors mentioned above act on the toxin translocation by altering the endosomal pH, H^+ pump or by acting on the ion channels. Even though this group of inhibitors have very narrow therapeutic window, they can be very effective in combination with the inhibitors against the endopeptidase activity. By targeting a general cellular process such as acidification of endosomes, these inhibitors are effective against almost all serotypes. However, at higher concentrations they alter the general cellular physiology. Thus, a major problem with such inhibitors is their toxicity at concentrations needed for their effectiveness. A way for the effective use of such inhibitors would be to design a way to target them to intoxicated cells selectively.

3.2.3 Non-peptidic inhibitors for the intracellular action

The third and perhaps the most important step of the BoNT mechanism is the intracellular endopeptidase activity, responsible for SNARE protein cleavage resulting in the blockage of neurotransmitter causing muscle paralysis. The HEXXH motif that is characteristic of the catalytic site of Zn endopeptidase is conserved. It is critical to find inhibitors against LC endopeptidase activity because it is the only strategy that may provide remedy for post-exposure conditions.

Several research groups have screened and developed small molecule drug candidates against post-entry intracellular BoNT/A endopeptidase activity. The compounds identified so far fall into different categories – non-peptidic small molecules (PSMs) with multi-aromatic rings and a keto

group [56-59] and PSM primarily based on BoNT/A substrates [60,61].

In search for non-PSM therapeutics, many commercially available real or virtual small medicinal molecule libraries have been screened. Screening of such libraries requires a robust assay that could be adopted for high-throughput operations. Such assays have been developed for screening against catalytic LC domain of BoNT [56,62,63] making it feasible to find inhibitors for endopeptidase activity of BoNT by screening libraries of compounds.

By screening diversified small molecule non-peptidic inhibitor (SMNPI) libraries, potential drug-like compounds can be identified for further confirmation and rational design to improve the potency. This strategy has been used for screening inhibitors against BoNT [57,64]. The pharmacophore design approaches have been identified from screening libraries [64]. The three-zone pharmacophore model for BoNT/A LC inhibitors was generated by using *in silico* SMNPIs for BoNT/A LC. Molecular modeling studies had predicted the pharmacophores based on the structures of some lead compounds. BoNT/A LC binds up to two biaryl/triaryl aromatic components of the inhibitors (two planes, A and B). Those two planes can act as scaffolds for positioning other components of the pharmacophore if distance between centroids of the two planes deviates from the optimum of ~ 6.5 – 9.5 Å. For plane A, presence of a heteroatom is also required, which may serve to either directly engage in an interaction with the catalytic zinc or potentially replace the water molecule used by zinc during substrate lysis. Two hydrophobic moieties (C and D) are key components of the common pharmacophore. They correspond to binding subsites of BoNT/A LC and facilitate hydrophobic collapse between inhibitors and the enzyme. The last component of this proposed pharmacophore is a positive ionizable substituent (E), which may participate in electrostatic interactions with enzyme residues and aid in solubilizing inhibitors bound within the BoNT/A LC substrate binding cleft [64]. Recently, 3D overlaying of most potent SMNPI analog with selected structurally diverse SMNPIs resulted in refinement of the 3-zone pharmacophore for BoNT/A LC [65]. By screening National Cancer Institute's (NSC) library for BoNT/A LC, indole-based bis-amidine inhibitor NSC 240898 was identified as a lead compound [66]. Based on the structure of this compound, two types of analogs have been synthesized and shown to inhibit LC endopeptidase activity by fluorescence resonance energy transfer (FRET)-based and high pressure liquid chromatography (HPLC)-based assays [67].

In another approach, synthesis-based computer-aided molecular design has led to an improved, serotype-selective, small-molecule BoNT/A endopeptidase inhibitor. This optimization was guided by multiple molecular dynamics simulations of the zinc-containing endopeptidase complexed with inhibitor or by using the cationic dummy atom (CaDA) approach. The results offer an improved template for further optimization of BoNT/A endopeptidase inhibitors and demonstrate that the CaDA approach is useful for both design and optimization of zinc protease inhibitors [58,68,69]. Pretreatment with compounds identified with this approach, such as F4H, H3H and F3A, showed prolongation of the mice death time with partial protection [69].

Similarly, virtual screening was performed by computationally docking compounds of NSC and ChemBridge databases into the active site of BoNT/A LC, identifying several quinolinol-based SMNPIs. The most potent inhibitors of this group are CB 7969312 and NSC 84087(7-((2-methoxyanilino) (phenyl) methyl)-8-quinolinol), reported by two different research groups [70,71]. Very recent *in silico* screening of the NIH library of ~ 350,000 compounds identified fungal metabolites, chaetochromin A and talaroderxines A as BoNT/A inhibitors [72]. *In silico* screening certainly reduces the cost and efforts in identifying potential inhibitors. However, BoNT biology and chemistry has been very unique and elusive, so typical assumptions made in algorithms applied for screening compounds may need further refinement based on the biophysical, biochemical and physiological knowledge.

Experiment-based screening of 16,544 ChemDive3 compounds identified 0831 – 1035 compound with a IC_{50} value in low μM range *in vitro* and *ex vivo* assays with primary cell culture [56]. It is a chalcone with one furan ring on each side of keto group separated by an alkene group. It is a mixed mode noncompetitive inhibitor, with a K_i value of 9 μM for BoNT/A LC. Another compound, L-arginine hydroxamate, is known to be a weak inhibitor of toxin with IC_{50} value of 60 mM [73]. Screening of a random 150-member hydroxamate library identified a small-molecule inhibitor of BoNT/A-LC, 4-chloro-cinnamic hydroxamate [59], and 2,4-dichlorocinnamic hydroxamic acid was obtained from subsequent structure–activity relationship analysis to improve the biological activity [74]. These compounds have been shown to have *in vivo* efficacy in mice and Neuro-2A cell-based assays and show no toxicity. These compounds were found to be competitive inhibitors of BoNT/A endopeptidase. The crystal structure of truncated LC (1 – 424) with each of the two hydroxamate inhibitors showed binding with the cinnamyl side chain orientated toward the 370 loop of BoNT/A LC, which forms a steep wall at one end of the active site cleft and constitutes part of the β-exosite. Janda *Group* has also identified two natural products D-chicoric acid and lomofungin against BoNT/A endopeptidase activity by interacting possibly with α- and β-exosites, respectively. In cellular bioassay with rat primary neuronal cell model, it has been demonstrated that lomofungin can protect SNAP-25 cleavage by LC/A [75]. Chicoric acid is a modest noncompetitive inhibitor for LC of A and B serotypes [76,77].

7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin (ICD 1578), a human leukocyte elastase inhibitor was reported to inhibit LC B. ICD 1578 inhibition was demonstrated using the FRET-based assay with the IC_{50} value of 27.6 μM [78]. Bis(5-amidino-2-benzimidazolyl) methane (BABIM) is a potential Zn metalloprotease

inhibitor. The HPLC-based *in vitro* assay determined the K_i value of BABIM as 1.2 μM, whereas that of the keto BABIM as 0.8 μM. BABIM is a weak Zn chelator than 1, 10 phenanthroline, but a better inhibitor for the LC B [79]. The crystal structure of the BoNT/B in complex with BABIM shows that the active site residues rearrange in the presence of the inhibitor and the active site zinc is progressively removed. The inhibitor partly occupies the site where the substrate would bind and block it from binding to the toxin [80,81].

So far, most efforts of developing inhibitors have been made with BoNT/A, with only some efforts for BoNT/B, leaving other BoNT serotypes unaddressed. None of the inhibitors identified have been advanced beyond hit and lead compounds. For developing them further into drug candidates, these compounds must be effective in cellular assays and *in vivo* conditions. Many of the identified compounds have not shown consistent efficacy under *in vitro*, *ex-vivo* (cellular), and *in vivo* conditions [74]. Furthermore, only limited information is currently available on toxicity, solubility and absorption, distribution, metabolism, and excretion (ADME) profile. It will take a great sustained effort on the part of a team of researchers to develop the lead compounds into drugs – a process that has not been initiated as yet.

3.3 Peptidic small molecules

Peptide-based inhibitors are designed as agonists using pharmacophoric groups involved in substrate binding. Detailed crystal structures of BoNT and BoNT complex with the substrates have been resolved for BoNT/A and BoNT/B. These 3D structures provide critical information about the molecular mechanism of BoNT action as well as information on how to design the inhibitors against them [82-87].

In an early approach of using substrate to inhibitor strategy, Schmidt discovered several inhibitors, including 2-mercapto-3-phenylpropionly-RATKMAL (K_i -330 nM) [60,88] and CRATKLM (K_i -1.9 μM) for the BoNT/A LC [89]. Molecular docking and modeling of the lead peptides with BoNT have allowed identification of pharmacophores for designing effective peptidomimetics as inhibitors of BoNT [66]. The docked conformation of 2-mercapto-3-phenylpropionyl-RATKML was then used to refine pharmacophore for botulinum serotype A LC inhibition. As a result of the pharmacophore characteristics thus recognized, a small-molecule inhibitor NSC 240898 was identified as a new lead molecule [66]. Co-crystallization of BoNT/A LC with an uncleavable portion of the substrate peptide, ^{197}Q-RATKM^{202}, defined detailed interaction sites, but the peptide itself was not sufficiently effective in inhibiting the BoNT/A endopeptidase activity (IC_{50} value of ≥ 94.9 μM) [90]. Further refinement of the substrate peptide, ^{197}Q-RATKML^{203}, resulted in peptidomimetic inhibitor I1 with a K_i of 41 nM for BoNT/A [81], thus indicating there is further scope of improvement with peptidic inhibitors.

Using similar approach, peptidic inhibitors have also been identified for BoNT/B and TeNT. Both BoNT/B and TeNT cleave the same peptide cleavage site, 76Gln-Phe77, of vesicle associated membrane protein (VAMP) (also known as synaptobrevin). The pseudopeptides mimicking the sequences of BoNT/B and TeNT that may interact with the synaptobrevin sequence Gln^{76}-Phe^{77} have been designed as potential inhibitors against BoNT/B and TeNT [91,92]. 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 is the left of the cleavage bond and S_1' and S_2' are at the right). Using -amino thiol derivatives to replace the side chain at the S_1 has led to a strong inhibitor against the endopeptidase activity of BoNT/B with a K_i value of 20 nM [91].

Another approach to develop peptide-based peptide inhibitors has targeted the SNARE motif of the BoNT substrates. Different serotypes of BoNT cleave specific peptide bonds present on one of the SNARE proteins. The specific cleavage is governed not only by the specific residues present at the cleavage site but also by the presence of a negatively charged deca-residue conserved motif, called SNARE motif, present in all the SNARE proteins [93]. [XH- - XH-XHP] is the general formula for the SNARE motif, where X is any amino acid, H is a hydrophobic amino acid, and P is a polar amino acid residue. The peptide V1 and V2 are based on VAMP; S1 – S4 are based on SNAP-25; X1 and X2 are based on syntaxin. Targeting this motif may prohibit binding of the BoNT to its substrate, resulting in the blockage of the proteolytic action of BoNT. Among all synthetically made peptide S3, a SNAP-25 derived peptide, and V2, a VAMP-derived peptide do not affect neuroexocytosis but were found to prevent the blockage of acetylcholine release in *Aplysia californica* neurons induced by BoNT/A and /B [93]. Thus, it can be hypothesized that the combination of both BoNT serotype active site inhibitor and SNARE protein-based peptide has potential to reverse the action of BoNT. Since the acidic residues are involved in the enzyme's active site and its vicinity and since arginine is located at the P1' position of the SNAP-25 substrate, several basic peptides have been optimized as effective competitive inhibitors (the most potent peptide, RRGF, with its IC_{50} as low as 0.9 μM and K_i of 358 nM) of BoNT/A [81,94].

Using the information on substrate peptide, ^{197}Q-RATKML^{203} and the basic peptide, RRGF, a dual combinatorial molecule was designed, with a K_i of 638.1 ± 92.0 nM, against BoNT/A LC in an effort to improve the effectiveness. However, this approach did not yield the desired result, owing to less favorable hydrophobic interactions of the peptide with the BoNT/A LC active site [95].

A combination approach of using SNARE motifs, substrate cleavage sites and zinc chelating amino acid residues have also been attempted, although its full potential has not yet been realized in designing comparatively effective inhibitor candidates [96]. Hinge peptide mini-libraries containing the structure of acetyl-X1-X2-linker-X3-X4-NH_2 or X1-X2-linker-X3-NH_2 (where X1, X2, X3 and X4 are one of the six amino acids; Asp and Glu are the conserved amino acids of SNARE motif; Gln

and Arg are cleavage site of BoNT/A and His and Cys are the zinc chelators) with 4-aminobutyric acid as a linker have been used to identify leads for BoNT inhibitors [92]. The result of these studies indicated that the most potent BoNT/A LC inhibitors with 75% inhibition had a general formula of Ac-X_1M_2-Linker-X_3C_4-NH_2 and C_1-linker-X_2S_3. Similarly, peptides with Ac-X_1C_2-Linker-X_3C_4-NH_2 and C_1-linker-X_2F_3 showed > 80% inhibition for BoNT/B LC under *in vitro* assay conditions [96].

Peptide-based drugs have two major limitations, namely, poor bioavailability and short duration of action. Therefore, the peptidomimetics should be designed based on the pharmacophoric groups responsible for the agonist activity. This approach has been applied in designing peptidomimetics against at least BoNT/A endopeptidase [57]. However, stability and bioavailability of such peptidomimetics are yet to be tested and verified. More work is obviously needed for further refinement of the inhibitor candidates and designing them for resistance against proteases to ensure their stability and bioavailability.

3.4 Aptamers

Aptamers are unique, single-stranded oligonucleotides (DNA or RNA) that bind to their targets with very high affinity and specificity. Technically, aptamers could be isolated against any protein known to mankind [97,98]. In solution, aptamers form intramolecular interactions that fold the molecule into a unique 3D structure, providing the basis for high affinity and specificity toward 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 systematic evolution of ligands through exponential enrichment (SELEX) [97] to generate a pool of DNAs or RNAs that have been enriched for those that bind to the target of interest (**Figure 2**).

Aptamers have high binding affinity and specificity to their targets (comparable to antibodies). A wide variety of modifications of aptamers can be applied for diverse applications. The robust structural stability and reversibility make aptamers easy to store and formulate.

Compared to antibodies, aptamers present several advantages for *in vivo* therapeutic applications: they have low toxicity, and more importantly, there are no reports of aptamer-induced immune response, which is one of the major limitations of antibody-based therapy. Due to small size (about 10 kDa, compared to 150 kDa antibodies), aptamers are likely to produce better tissue penetration [99]. 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 [100].

The advantages of aptamers make them attractive therapeutic countermeasures for biothreat toxins, including BoNT. Our group pioneered in this area and first reported the identification of three RNA aptamers that bind to a LC of BoNT/A, and inhibit the endopeptidase activity of BoNT/A [34]. The IC_{50} values for those aptamers are in the low nanomolar range. Enzyme kinetic studies showed that they exhibit noncompetitive inhibition of the enzymatic function of BoNT/A [34]. More important, our preliminary data have shown partial protective effects of those RNA aptamers on mouse models against BoNT [101]. Due to their low toxicity, aptamers open a new avenue for much needed antidotes against botulism.

BoNTs, like many other A-B type toxins, have their targets at the intracellular level. Although it is straightforward for aptamers to target the extracellular targets, for intracellular targets, like BoNTs, a special delivery system is needed for aptamers to reach their intracellular targets and to make them effective therapeutics to reverse paralysis caused by BoNT and accelerate the recovery. The unique property of BoNT provides an effective way of transportation through cellular barriers. HC of the BoNT has high binding specificity to neuronal cells with high binding affinity, and yet, by itself does not pose any toxicity. This provides a highly specific binding vehicle for the intoxicated cells [102,103]. Our laboratory has shown that HC of BoNT can effectively deliver the cargo into neurons through receptor-mediated endocytosis (**Figure 3**) [104].

Liposomes and other biodegradable polymeric nanoparticles have been used for delivering nucleic-acid based therapeutics, such as RNAi [105]. By functionalization of the surface of both liposomes and polymeric nanoparticles with BoNT, HC will provide a unique targeted drug delivery vehicle for neuronal cells, and this vehicle can be used for delivering therapeutics into BoNT-intoxicated cells as a countermeasure against botulism. The robustness of aptamers' structural stability makes the encapsulation of aptamer-based cargo more feasible. This will help the development of aptamers as true therapeutic countermeasures against biothreat toxins. Exploration of aptamers as therapeutics offers a new avenue for developing specific treatment for botulism.

4. Conclusion

BoNT is a unique molecule with both constructive and destructive features for public health. As a biowarfare and potent biothreat agent, on one hand, and as a therapeutic and cosmetic agent, on the other, it poses an exceptional challenge for public health management. Its massive toxicity has potential for extensive damage to public health, necessitating development of countermeasures and antidotes. Typically, vaccination is a preventive measure for such grave situation. However, the medical use of BoNT against neuromuscular disorders makes life-long immunization against BoNT less desirable, thus leaving therapeutics as the only option against botulism. Thus, development of antidotes, including therapeutic agents in the form of inhibitors of the BoNT endopeptidase activity is acutely critical.

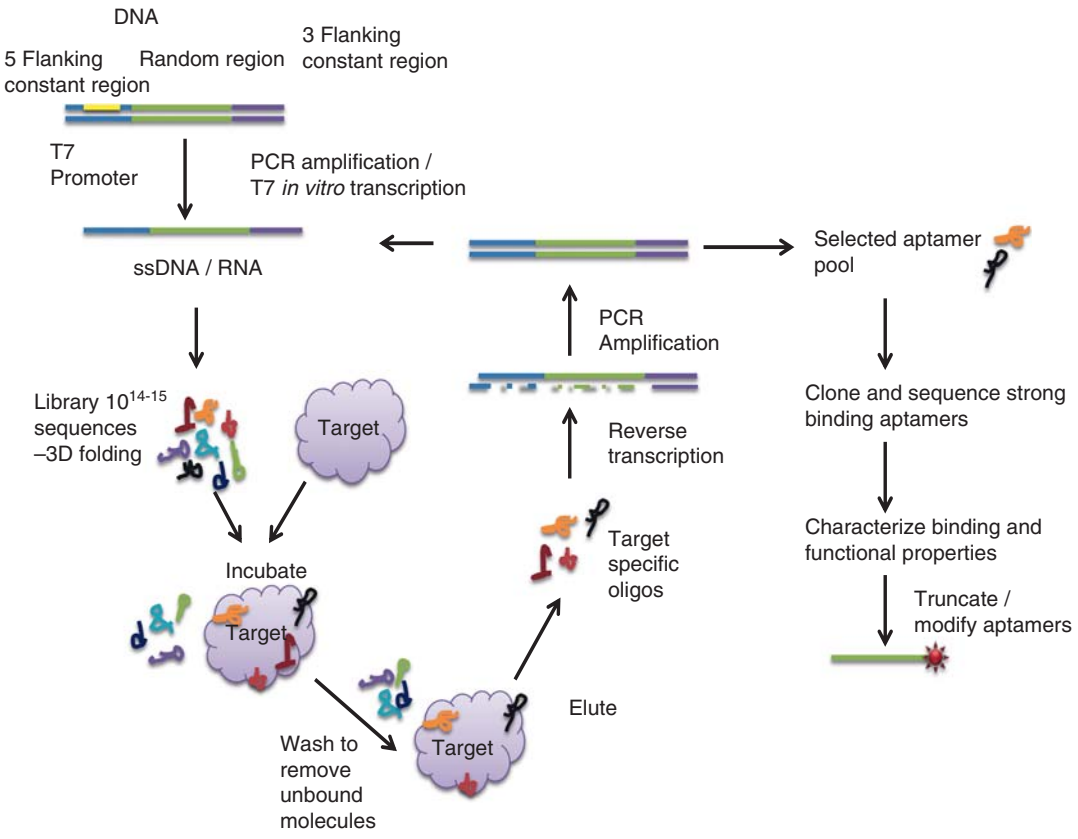

Figure 2. Schematic representation of systematic evolution of ligands through exponential enrichment. A library of DNA or RNA molecules is incubated with the protein target, and those that bind to it are separated from the rest. The bound sequences will be amplified by polymerase chain reaction (PCR) (reverse transcription 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 toward the sequences with strongest binding affinity with the protein target [121].

A series of functionally diverse groups of inhibitors have been identified over the past decade or so (**Table 1**). However, therapeutic candidates reported so far have several limitations, including limited therapeutic window, short half-life of the molecule, poor cell permeability, less bioavailability, toxicity of the molecules and poor ADME profile.

Inhibitors and drugs targeted at toxin binding, internalization and translocation have a very limited therapeutic window and thus have limited scope. Antibodies even with limited therapeutic window are the only effective antitoxins currently available for removing circulating BoNTs in patients. The US government has therefore invested millions of dollars in stockpiling both equine polyclonal and humanized mAbs. Small-molecule inhibitors have been developed to disrupt toxin internalization and translocation by disrupting the acidification process of the endosomes. However, a relatively high concentration is needed for their effectiveness, and they affect many normal physiological processes, making their safety profile much less favorable. In addition, the therapeutic window is also relatively short when targeting this step. Therefore, the most sought-after step for designing antidotes against botulism is its intracellular endopeptidase activity, with a target to reverse the flaccid paralysis. However, no successful candidates have been developed as yet. Several inhibitor candidates have been identified within the past decade, but most of these did not show consistent results through *in vitro*, cellular, *in situ* and animal testing. For example, Eubanks *et al.* identified several inhibitor candidates from screening with *in vitro* endopeptidase activity [74], which were not effective in cell-based assay, and those effective in cell-based assay showed poor effectiveness in mouse bioassay.

5. Expert opinion

There are several major problems in advancing the molecules further as therapeutics against botulism. i) The molecules have shown unusual toxicity either at the cellular or animal levels. ii) Inconsistent efficacy has been observed under *in vitro*, cell and animal assay conditions [74]. iii) Usual problems of bioavailability with PSM group. Part of the reasons

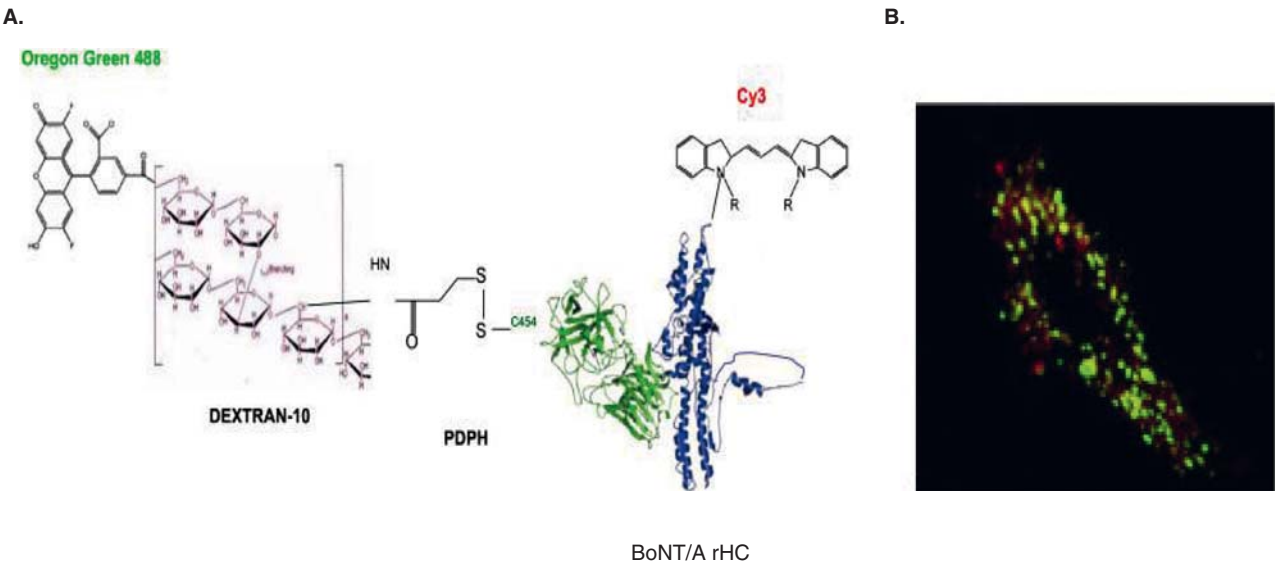

Figure 3. Drug delivery vehicle consisting of recombinant HC linked with 10 kD dextran and Oregon Green 488 (green), and Cy3 (red) on rHC. (A) shows that 200 nM drug delivery vehicle was delivered into mouse spinal cord neurons, as demonstrated by both green fluorescence and red fluorescence **(B)**.
Reproduced from [104] with permission of BioMed Central.

Table 1. Summary of therapeutic approaches to BoNT inhibition.

Therapeutic (ref.)	Efficacy	Limitations
Anti-botulinum neurotoxin (BoNT) monoclonal antibodies (mAbs) [35]	Effective in mice (*in vivo* toxin neutralization when premixed with BoNT/A prior to injection)	Three mAbs required (oligoclonal) for adequate potency; limited post-exposure utility
Receptor decoys [112,113]	Effective in nerve assays when premixed prior to contact	Coadministration of gangliosides required; limited post-exposure utility
Heavy chain inhibitors [43,44]	Effective in isolated mouse diaphragm muscle twitch model	Mechanism unclear; associated cytotoxicity of antimalarials; no post-exposure protection
Light chain inhibitors – peptides [60,92,114,115]	Efficacy demonstrated *in vitro* only	Non-drug-like molecules with poor ADME features
Light chain inhibitors – non-peptidic small molecules [57,59,64,78,80,91,116,117]	Efficacy *in vitro* and in neuronal cell culture [118] or synaptosomes [119]	Higher potency with suitable ADME properties needed. Inconsistent efficacy in cell, animal and *in vitro* assays

for these problems has been the use of either inappropriate substrate (peptide-based vs full-length protein substrate) or inappropriate form of the BoNT/A enzyme (several forms of truncated BoNT/A LCs) during the screening process. Even a few amino acid residue difference in the length of the LC results in a substantial difference in the catalytic constant K_{cat} [106]. Using different form of substrate (peptide, full-length, fusion protein) also makes substantial difference in the binding behavior. The inconsistent approaches used in the early stage of development have diminished the effort of finding effective antidotes and have wasted precious resources and time.

A major challenge in developing biologically consistent inhibitor against the endopeptidase activity of BoNT is the structurally flexible molten globule-like structure that the endopeptidase adapts under physiological conditions [107,108]. While the structure of several serotypes of BoNT (including the most potent type A) has been resolved by X-ray crystallography, the differences between solution structure and crystal structure make it difficult to use crystal structure as the sole guide for designing and modifying the inhibitors [66,73,84,109-111].

In order to use the resources more effectively to speed up the development of antidotes, a streamlined process should

be applied by employing standardized reagents and assays (including substrates and toxins), with close collaborations between medicinal chemists and structural/biochemists with extensive knowledge on BoNT. The native toxin is preferred during the development, even though that would limit the available laboratories to carry out such research due to the restrictive regulations of tier 1 select agent for research with BoNT. During the early development, recombinant LC could be used, but the full-length LC should be employed, as it represents the enzymatic domain of the native toxin. Similarly, although peptide-based substrate can be used in early screening, full-length protein substrate, as well as native toxin, need to be used for validation of the leads at earlier stage.

Crystal structure of BoNT can be used to help in designing and modifying the lead inhibitors. However, precautions have to be taken to ensure the predictions are confirmed in solution-based assays. To this end, molecular dynamic modeling of toxin and its lead inhibitor candidates in solution state may provide more insight during inhibitor design and modification. *Ex vivo* cellular assays and animal models also need to be validated for confirmation studies on the leads and for preclinical study.

A major challenge for drug development (including the antidotes against botulism) is to increase the bioavailability of the molecule. Extensive formulation optimizations need to be involved as early as leads are identified. This is especially important for BoNT, as the target is at the intracellular level. The nanosizing can increase the solubility, dissolution, permeability and bioavailability, and the targeted delivery to intoxicated neurons is critical for effective antidotes against botulism.

Those streamlined approaches would be difficult for any single laboratory. Pharmaceutical companies may not have enough motivation to get into this area, because of the rarity of botulism. A more feasible approach could be to form a comprehensive consortium, including experts on BoNT, medicinal chemists, pharmacologists, structural biologists, formulation scientists and clinicians. By using an array of comprehensive approaches and collaboration from the multidisciplinary consortium, effective antidotes against botulism will be on the horizon soon.

Declaration of interest

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