Botulinum Neurotoxins as Biothreat Agents

Theresa J Smith1, Virginia I Roxas-Duncan1 and Leonard A Smith∗

1Integrated Toxicology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702
2Office of the Chief Scientist, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702

Abstract
Botulinum neurotoxins (BoNTs), a family of proteins produced by the bacterium Clostridium botulinum and other clostridial species, are considered to be the most potent toxin known, and a potential biological weapon. BoNTs cause botulism, a serious and life-threatening illness in humans and animals. There are seven immunologically distinct serotypes of BoNTs, each with multiple unique subtypes/genetic variants. BoNT potency and diversity present challenges for detection, diagnosis, treatment, and development of countermeasures. This article provides an overview of the range, diversity, and potency of BoNTs, and how these features might affect their use as biothreat agents.

Keywords: Botulinum neurotoxins; Biothreat; Diversity; Assays; Antitoxins

Abbreviations: BoNT: Botulinum Neurotoxins; PCR: Polymerase Chain Reaction; SNARE: Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor; FRET: Fluorescence Resonance Energy Transfer; PFGE: Pulsed Field Gel Electrophoresis; AFLP: Amplified Fragment Length Polymorphism; MLST: Multi-Locus Sequence Typing; MLVA: Multiple-Locus Variable number tandem repeat Analysis; VNTR: Variable Number Tandem Repeat; SNP: Single Nucleotide Polymorphism

Introduction
Botulinum neurotoxins (BoNTs) are extremely potent protein toxins produced by the bacterium Clostridium botulinum and related clostridial species. The bacteria are anaerobic spore-formers that are relatively easy to culture, and the toxins that are produced are stable in complexed form, but somewhat heat labile and easily destroyed during autoclaving or cooking.

The toxins cause a neuroparalytic syndrome characterized by a descending, bilateral flaccid paralysis that can be fatal due to respiratory arrest. Naturally occurring intoxications can arise within hours of ingestion of preformed toxin (food borne botulism). They may also be due to the elaboration of toxins after colonization of contaminated wounds and abscesses with BoNT-producing clostridia (wound botulism), or from organisms that have colonized the intestinal areas of susceptible infants (infant botulism) or adults (adult toxicoinfections). Intoxications may also occur after deliberate injection by injection or inhalation.

BoNTs are considered to be category A select agents because they have great potential for adverse public health impact with mass casualties, they require broad-based public health preparedness efforts, and they have moderate to high potential for large-scale dissemination [1]. These factors also make botulinum neurotoxins primary candidates as bioterrorism or biothreat agents.

History
Botulism has probably occurred sporadically since food storage methods involving drying, salting, pickling, or smoking were introduced. However, accurate descriptions of intoxications were not available until early in the 19th century [2]. Justinus Kerner’s monographs on “sausage poison” in 1820 and 1822 are generally considered the first accurate and complete descriptions of the disease [2]. The connection of botulism with a biological organism occurred with the isolation of a gram positive bacillus from ham which produced a substance that was toxic in animals [3]. The first descriptions of botulism that were not associated with meat or fish were published in 1904, after an outbreak in Darmstadt, Germany that was due to canned white beans [4]. Subsequently it was noted that, while most botulism outbreaks in Europe were traced to meat or fish products, most food borne cases in the US were due to vegetables. Several severe outbreaks of botulism occurred in the US from late 1919 to early 1920, associated with the consumption of commercially canned ripe olives. These incidents were particularly deadly, with an overall fatality rate of 78%. The severity and widespread nature of these cases prompted the establishment of strict guidelines concerning commercial food canning and intensified research in this area [5]. These highly publicized outbreaks, plus others occurring in Europe over the following decade [6], brought attention to the deadly nature of this toxin, which may have prompted some governments to pursue this toxin as a biological weapon.

State-sponsored programs for the development of BoNTs as biological weapons have been instituted in Canada, Germany, Iraq, Japan, the UK, the US, and the USSR [7,8]. However, to our knowledge only Japan has actually tested such weapons on humans. General Shiro Ishii, head of the infamous Japanese Unit 731, admitted to testing botulinum toxin as a weapon in the 1930s by feeding Manchurian prisoners botulinum toxin-laced food [9].

In early 1944, Allied intelligence uncovered plans indicating that Germany was planning to use BoNT as a weapon against invading forces. This led to the initiation of a US program that included researching the use of BoNTs as weapons [8]. While the US program was abolished in 1969, the Soviet Union continued pursuing BoNT as a biological weapon, despite their signing the Biological and Toxin
Weapons Convention agreement in 1972. This research was continued until the fall of the Soviet Union in the early 1990s.

In 1992, Iraq admitted to the United Nations that they had produced approximately 19,000 liters of concentrated BoNT; some of this material had been loaded into military weapons [7].

State-sponsored programs were not the only sources of concern. In the 1990s, the Japanese cult Aum Shinrikyo cultured C. botulinum strains to produce toxin, which they disseminated on at least three occasions in Tokyo using aerosol release. The toxin was produced by a clostridial strain that was reportedly isolated from northern Japan. These attempts were not successful, apparently due in part to a lack of toxin production in the cultures [10].

**Diversity**

Clostridium botulinum organisms produce a series of neurotoxins that are closely related in structure and function, but surprisingly diverse in genetic and amino acid sequences. There are seven toxin serotypes (A-G). Four of these, BoNT/A, /B, /E, and /F, are considered to be human pathogens; the other three serotypes are not generally thought to be human pathogens, even though there have been a few human cases associated with these toxin types.

BoNT serotypes differ in their host range, potency, and course of intoxication. Host susceptibility differences that are related to toxin differences are known. For example, humans are very susceptible to BoNT/A and BoNT/B, but appear to be somewhat resistant to BoNT/C, /D, and /G. Rats and chickens are susceptible to BoNT/A, but resistant to BoNT/B [11]. Paralytic effects due to serotype A can last for many months, but effects due to BoNT/B or /E diminish after a few weeks. Infant botulism cases caused by BoNT/F can occur in very young babies (less than one week old); in contrast to BoNT/A or /B, these intoxications tend to resolve rapidly, especially after treatment with specific antitoxin.

Each serotype can be divided into two to eight subtypes/genetic variants that differ by as little as 1% or as much as 36% in amino acid sequence (Table 1). The toxins are expressed in one of six different clostridial species (C. botulinum groups I-III, C. argentinense, C. baratii, and C. butyricum) [12].

C. botulinum group I organisms may produce any one of three toxins that are usually associated with human botulism (serotypes A, B and F). In addition, members of this group may concurrently produce multiple toxins, as in the case of BoNT/Ab, /Ba, or /BF. The toxins produced by Group I species are generally of higher yields and toxicity in culture than those produced by other species. One of these strains, BoNT/ A1 Hall, has been reported to yield toxin concentrations of 1,000,000 mouse i.p. LD<sub>50</sub>/ml from crude cultures under optimal conditions [13]. Toxins produced by Group I strains also show the greatest subtype diversity. BoNT/A subtypes differ by as much as 15% at the amino acid level; BoNT/F subtypes are even more diverse. Amino acid differences of 26% are seen among the four BoNT/F subtypes produced by Group I clostridia (BoNT/F2-F5) and there is 36% difference between BoNT/F5 and BoNT/F7, which is produced in C. baratii strains (Table 1). This level of diversity is similar to that seen between the BoNT/E and BoNT/F serotypes (Table 2). Some subtype differences also result in host range differences. Mammals, especially cattle, are extremely susceptible to BoNT/C1 but resistant to BoNT C/D. The reverse is true in birds. Catastrophic die-offs in waterfowl due to BoNT C/D have been seen in waterfowl and other migratory birds [14]. Subtype variation has also impacted the effectiveness of BoNT countermeasures [15-17].

<table>
<thead>
<tr>
<th>BoNT subtypes</th>
<th>% identity</th>
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<tbody>
<tr>
<td>A1-A5</td>
<td>85.0-97.1</td>
</tr>
<tr>
<td>B1-B6</td>
<td>92.7-98.4</td>
</tr>
<tr>
<td>C-C/D</td>
<td>75.8-76.5</td>
</tr>
<tr>
<td>D-D/C</td>
<td>72.7-76.4</td>
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<tr>
<td>E1-E8</td>
<td>94.7-99.0</td>
</tr>
<tr>
<td>F1-F7</td>
<td>63.8-97.0</td>
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</tbody>
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**Table 1.** Amino acid residue identities among BoNT subtypes.

<table>
<thead>
<tr>
<th>BoNT A1</th>
<th>BoNT B1</th>
<th>BoNT C1</th>
<th>BoNT D</th>
<th>BoNT E3</th>
<th>BoNT F1</th>
<th>BoNT G</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoNT A1</td>
<td>-----</td>
<td>37.5</td>
<td>30.4</td>
<td>31.3</td>
<td>37.7</td>
<td>38.7</td>
</tr>
<tr>
<td>BoNT B1</td>
<td>-----</td>
<td>30.8</td>
<td>32.6</td>
<td>35.9</td>
<td>37.4</td>
<td>57.1</td>
</tr>
<tr>
<td>BoNT C1</td>
<td>-----</td>
<td>51.5</td>
<td>30.9</td>
<td>30.9</td>
<td>32.5</td>
<td></td>
</tr>
<tr>
<td>BoNT D</td>
<td>-----</td>
<td>31.7</td>
<td>32.7</td>
<td>34.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BoNT E3</td>
<td>-----</td>
<td>62.8</td>
<td>36.4</td>
<td></td>
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<tr>
<td>BoNT F1</td>
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<tr>
<td>BoNT G</td>
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**Table 2.** Percent identity of amino acid residue among BoNT serotypes. The most conserved serotypes are in bold type.

C. botulinum group II organisms produce nonproteolytic BoNT/B, /E and /F. Toxin yields and specific activities are much lower than with the Group I organisms. However, these organisms have the capability to grow and express toxin at low temperatures (3.3-10°C) [18-20], making vacuum-packaged foods stored at refrigerated temperatures vulnerable if contaminated with botulinum spores.

Group III organisms produce BoNT/C and BoNT/D toxins, which are not generally considered to be human botulism threats. Information on toxin yields for these serotypes is not readily available. However, recent calculations of BoNT/C and /D specific activity per mg of toxin indicate that the two BoNT/C subtypes were not as potent in mice as other toxin serotypes, but that BoNT/D subtypes may be the most potent of all toxin types in mice [21]. Group IV organisms produce BoNT/G, which is not considered to be a human pathogen. Culture yields may vary, depending on the strain used, culture conditions, and number of culture passages [22].

**Range**

Environmental samples from Argentina [23], Brazil [24], China [25], Europe [26], Greenland [27], Hawaii [25], Indonesia [28], the US [29], and Venezuela [30] have all yielded BoNT-producing clostridial strains. BoNT-producing organisms have been found on every continent with the exception of Antarctica, including remote places such as Korea (unpublished – NCBI accession no. EF506573), Mauritius [31], northern Scotland [6], Tasmania [32], and Tierra del Fuego [33]. With such a widespread geographic range, it should not be difficult to isolate BoNT producing clostridia from most areas of the earth.

There have been 1,835 cases of naturally occurring botulism in the US from 1950 to 1996. Of these, 921 were associated with BoNT/A, 818 were associated with BoNT/B, 67 cases were associated with BoNT/E, and 23 were associated with BoNT/F [34]. Approximately 95% of all naturally occurring cases in the US can be attributed to either BoNT/A or BoNT/B. This predominance of serotypes A or B as causative toxins in botulism appears to be a widespread phenomenon; for example, the major serotype associated with food borne cases in Argentina [33], France [35], Poland [36], the UK [37], and the country of Georgia [38] has been either type A or B. Worldwide, 98% of reported infant botulism cases have also been associated with either BoNT/A or /B [39].

Only three outbreaks attributed to BoNT/C have been recorded.
worldwide. One case was in California, where type C was isolated from the gastric contents of a person with fatal botulism [40]; the second outbreak involved contaminated mushroom paste in France in 1955 [41], and the third was an infant case in Japan [42]. A lone foodborne outbreak due to BoNT/D was reported in Chad in 1958 [43]. While BoNT/G has been isolated from autopsy materials of several persons that had experienced sudden unexplained deaths [44], no evidence was found that supported botulism due to type G as the cause of death.

The ready availability of BoNT/A and BoNT/B strains worldwide, coupled with their apparent potency as judged by their association with botulism cases, make these the most likely two serotypes to be used as bioterror agents.

There is also the problem of dissemination of standard BoNT-producing type strains that are used as controls in assay development or assessment of food preparation conditions. For example, the ATCC C. botulinum type strain, ATCC 27563, has been reported to reside within strain collections in Australia and New Zealand [45], Brazil [46], Finland [47], France [48], Iraq (US Dual-use Exports to Iraq and their Impact on the Health of Persian Gulf War Veterans, Hearing before the Committee on Banking, Housing, and Urban Affairs, United States Senate, May 25, 1994), Sweden (Culture Collection of the University of Goteborg), and the UK [48], as well as the states of Georgia [49], Maryland [50], Ohio [51], Pennsylvania [52], and Virginia [53] within the US. Tracing the origin of a possible bioterrorist event involving this strain would be challenging.

A further concern is the manufacture of BoNTs as therapeutic agents. A surge in manufacturing facilities that are outside of the US and Europe might provide access to less secure stocks of BoNTs, which could be used as biological weapons [54].

**Potency**

Botulinum neurotoxins are the most potent poisons known. Several picograms of toxin can be lethal for small animals, and several human deaths after tasting and spitting out contaminated food have been recorded [55].

Judging by their predominance in naturally occurring botulism cases, it would seem that BoNT/A and /B should be more potent than other toxin serotypes. This appears to be the case, as reports of estimated oral lethal doses in nonhuman primates have determined the minimum lethal dose for serotypes A and B to be 7-16 ng/kg, while estimates for other serotypes range from 50 ng/kg to 14 µg/kg (Table 3) [56].

However, the mode of introduction can be important. Estimated human lethal doses for BoNT/A have been calculated as 0.09-0.15 µg (per 70 kilogram person) when injected, 0.7-0.9 µg when inhaled, and ~70 µg when ingested [9]. Additionally, while BoNT/C is rarely implicated as the causative agent in foodborne botulism or toxicoinfections in humans, it has been reported that BoNT/C, when injected, produces an effect that is similar to BoNT/A in both intensity and longevity [57]. Thus, while intentional release of BoNT/C into the food supply would not be very effective, an attack using injected toxin, analogous to the assassination of the Bulgarian dissident Georgi Markov with ricin [58] might prove to be very effective.

**Economic and Agricultural Terrorism**

As we have learned from the Amerithrax incident, the effects of a bioterrorism incident are not limited to human illness and fatalities. These incidents can cause severe emotional distress and cost millions of dollars (it has been estimated that the clean-up costs following the anthrax incidents were more than $1 billion) [59]. Deliberate exposure of a civilian population to BoNT, while not having the issues of continuing infectivity, would have a significant effect on the health care delivery system. Costs after an aerosol exposure to BoNT in a major city have been estimated at $1-$8 billion [60,61].

The 2005 publication of a journal article predicting the possible poisoning of hundreds of thousands of people from a single point-source contamination of the milk supply with 10 g of BoNT [62] caused alarm among the scientific community. While these predictions were later found to be exaggerated [63], deliberate contamination of the milk supply remains a concern [64].

Accidental contamination of central food stocks for fur-bearing animals housed on small farms caused the catastrophic loss of over 50,000 animals [65]. Botulism outbreaks in cattle and other domestic animals may cause the deaths of hundreds of animals over the course of only a few days. Cattle are particularly susceptible to BoNTs. They are more than ten times more sensitive to BoNT/C than mice on a per kilogram weight basis [66]. Botulism outbreaks in cattle have been increasing over the past decade, which has also raised concern over possible outbreaks of human botulism due to contaminated dairy products [67]. Domestic fowl are also targets for botulism; fatality rates are similar to those seen in cattle [68,69]. Deliberate contamination of feed with BoNTs could cause heavy economic losses, especially in areas that are dependent on dairy farming and meat production.

**Detection and Identification**

The classical method of detection and serotype determination for BoNT has always been through the use of in vivo mouse neutralization assays [70,71]. While sensitive, this method requires a large amount of sample and takes several days, making it somewhat impractical in the age of rapid diagnosis/detection. Classic ELISA methods that use colorimetric endpoints have also been used. They are rapid, but not particularly sensitive [72]. Recently, several alternative methods have been developed that are rapid, require small sample volumes, and are equivalent in sensitivity to mouse neutralization assays.

Primary among these are PCR-based assays that are capable of detecting and amplifying toxin-specific DNA that is associated with the neurotoxins. Multiple assays have been developed that detect and serotype BoNTs directly from food matrices and clinical samples [73-78]. These assays can be completed in a matter of hours and, with the advent of multiplexing, tests for multiple subtypes can be run simultaneously using a single small sample. Limits of detection with these assays may be as low as 10 gene copies per reaction [77].

In vitro protein-based detection assays are also being developed that show great sensitivity and specificity. Summaries of various procedures and their limits of detection can be found in Baghran et al. [79] and Cai et al.[80]. ELISAs that utilize high-affinity antibodies and sensitive detector systems, such as digoxygenin or chemiluminescent reagents,
provide assays with sensitivities approaching that of mouse bioassays (50–400 pg) [81, 82] in different matrices and food samples. However, the sensitivity of these assays can vary depending on the choice of capture and/or detector antibodies.

An interesting merger of DNA- and protein-based methods is the immuno-PCR assay. The assay utilizes a capture antibody that binds toxin, with a biotinylated antibody as detector. The detector antibody is linked to a biotinylated reporter DNA fragment via streptavidin. PCR reagents and specific primers are added to the mix, and the resulting PCR products are reflective of the amount of toxin present in the sample. This assay is very sensitive, with reported limits of detection of 50 fg/ml [83]. A further development on this assay encapsulates the DNA within liposomes. The liposomes protect the DNA from DNAse during antibody binding and washing steps; release occurs during real time PCR. Reported limits of detection with this liposome PCR assay are a staggering 0.02 fg/ml BoNT/A [84], under ideal conditions using purified toxin.

Additional sensitive protein assays have been developed that are based on the enzymatic activity of the BoNTs. Each BoNT cleaves one of three target SNARE proteins (SNAP-25; synaptotagmin, or VAMP; and syntaxin) at a unique site. Multiple FRET assays are available that use fluorogenic reporter reagents containing peptides with cleavage sites for the toxin to be assayed [85-87]. Intact peptides are in a quenched state; cleavage results in an increase in fluorescence. As this is an enzymatic process, each toxin molecule may cleave many peptides, resulting in enhancement of the fluorescent signal.

Two different assays have adapted this method for testing clinical and food samples. The ALISSA (assay with a large immunosorbent surface area) utilizes a two-step procedure. The first step involves the binding of toxin to antibody-coated beads which are then washed to remove potential inhibitory substances, and the second step is a FRET assay [79]. Limits of detection for BoNT/A with this method were 0.5 fg/ml using spiked human serum or liquid food samples.

A second activity-based method is the Endo-PEP assay, which also involves an initial antibody/toxin-binding and wash step to concentrate the toxin and remove inhibitory substances. However, enzymatic activity is detected using mass spectrometry. Peptides containing the target sites for each of the BoNTs have been manufactured. The sizes (or masses) of the cleavage products from these peptides, which are unique for each serotype, are measured using MALDI-TOF mass spectrometry. The sensitivity and specificity of these activity-based assays make them appealing, as they are capable of identifying the presence of toxins of a specific serotype (or multiple serotypes in the case of bivalent toxin producers) from a variety of food matrices and clinical samples in a matter of hours with sensitivities that are below mouse bioassays [88].

More definitive identifications, such as strain or isolate level comparisons, require larger sample sizes and more time, whether using DNA-based or protein-based assays. Differentiation of BoNT-producing strains has been done using a variety of DNA-based methods [27,50,89-91]. PFGE methods for strain comparisons are useful but they require culture of the toxin-producing organisms [92]. AFLP and some SNP analyses require a source of pure, concentrated DNA, but MLST, MLVA (VNTR) and other types of SNP analyses can be done using DNA that is naturally found in conjunction with the neurotoxin.

Toxin subtype can be differentiated using mass spectrometry methods; these identifications require nanogram amounts of toxin, which may require culture of the organism [93,94].

In naturally occurring cases of botulism, the causative organism is often obtained from contaminated food or clinical samples, but this might prove difficult in a scenario where toxin has been deliberately released. Thus, the choice of screening assay and characterization methods might depend heavily on the amount and type of sample provided.

### Treatment and Countermeasures

Current treatment for botulism consists of supportive care and the administration of antitoxin. Case severity can range from minor changes in sight or voice [95] to extended periods of near-total paralysis with loss of respiratory and gastrointestinal function. Mild cases may require minimal care, but more severe cases could require weeks in intensive care or special hospital units. Occasional severe cases can be handled with a minimum of disruption, but an influx of severely intoxicated patients, such as might be seen after a deliberate release, would overwhelm the health care systems of most major metropolitan centers [60].

An investigational heptavalent botulinum antitoxin (HBAT) has recently been made available under an Investigational New Drug (IND) protocol for use in the treatment of non-infant botulism [96]. Pretesting for reactogenicity is required, and the product must be diluted and slowly administered to avoid serious adverse reactions. This product has been depsecticated to reduce its reactogenicity; however, the resulting Fab and F(ab')2 fragments are cleared from the system more rapidly than intact IgG, so that repeat dosing may be necessary [96].

BabyBig (Botulism Immune Globulin Intravenous (Human)), an FDA approved human antibody product, is used for the treatment of infant botulism [97]. As this product is made from human antiserum, the adverse side effects associated with administration of equine antitoxin have been eliminated. The product is indicated for the treatment of infant cases involving serotypes A and B (product insert, BabyBig, revised September 2009). It may also be effective against serotypes C, D, and E, but it is not effective against BoNT/E.

While these products make potent therapeutics, they are expensive to produce and lot-to-lot variability is common. Human and human-compatible monoclonal and polyclonal anti-BoNT antibodies are currently being manufactured to replace HBAT equine antibody preparations and BabyBig [98-102]. Combinations of recombinant human monoclonal antibodies specifically engineered for potent protection against multiple toxin serotypes and subtypes (oligoclonal antibody) have been developed [101,103]. These oligoclonal antibodies are extremely potent, and they can be inexpensively produced and custom-mixed, eliminating lot variability problems and adverse events associated with the infusion of large amounts of heterologous protein. An anti-BoNT/A product is currently in clinical trials to support licensure as an FDA-approved product; anti-BoNT/B and/or BoNT/E antibody products are in advanced development. Because of their high potency, these products can be administered as a bolus injection in much lower doses than antitoxin. In addition, the half-life of human antibodies in humans is much longer than heterologous antibody, so that it is possible to protect against intoxication for months with a single application. These characteristics could permit both prophylactic and therapeutic use of these antibody preparations. Vaccination confers possible lifelong immunity that eliminates the option of treatment with therapeutic BoNT products. On the other hand, annual or semi-annual injection of antibodies, which protect for a specified time period only, could be discontinued if no longer necessary, allowing individuals to benefit from therapeutic BoNT use.
A pentavalent toxoid vaccine was manufactured in the 1980s to protect laboratory workers and others who might be exposed to large amounts of botulinum neurotoxins. While the vaccine was shown to be effective against BoNT/A-E [104], it has been declining in immunogenicity and potency over the past decade. In addition, reports of injection site-related adverse reactions have been increasing. Because of these issues and the age of the product, the CDC has discontinued providing this vaccine and no longer recommends its use [105].

Newer vaccines have been developed that are based on large subunit fractions of the toxins. Second-generation vaccines based on LC-H 

or H 

domains are potent and effective against a variety of toxin subtypes [17,106,107]. Phase II clinical trials of a recombinant bivalent BoNT A/B H 

C to support FDA licensure have recently been completed [108].

Third-generation vaccines that employ nontoxic, catalytically inactive BoNT holotoxins are currently being developed [16,109,110]. These recombinant proteins have been genetically altered in their active enzyme sites, rendering them catalytically inactive but fully immunogenic. As the entire protein is represented, a larger number of epitopes are presented for immune recognition. This results not only in greater overall efficacy, but also improved protection against alternative toxin subtypes [16].

There are currently a large number of disorders that can be effectively treated using therapeutic BoNT preparations. However, vaccination can effectively neutralize these toxins, rendering them ineffective as therapeutics. To avoid this problem, a mucosal vaccine of vaccination can effectively neutralize these toxins, rendering them effectively treated using therapeutic BoNT preparations. However, toxin subtypes [16]. A/B HC to support FDA licensure have recently been completed [108].

Some natural products have also been reported to inhibit BoNT. Zou et al. [137] described the inhibitory effects of toosendanin, a triterpenoid derivative from the bark of Melia toosendan, against BoNT/A, /B and /E in monkeys. The ability of toosendanin and a more potent tetrahydroyfuran analog to selectively inhibit translocation of BoNT/A and /E LCs with subnanomolar potency has been elucidated [138]. A function-oriented synthesis (FOS) strategy has been applied to toosendanin analogs [139,140]. Other reported natural product BoNT inhibitors include the aqueous extract from stinging nettle (Urtica dioica) leaf [141] that showed inhibitory effects against BoNT/A LC, and the thearubigin fraction of black tea (Camellia sinensis) that reduced the onset of paralysis in mice due to intoxication by BoNT/A, /B and /E [142]. Lomofungin, a secondary metabolite that was first isolated from the soil-dwelling gram-positive bacteria Streptomyces lomodensis, was identified as a BoNT/A LC inhibitor from a high throughput screening (HTS) of a drug library [143]. Lomofungin displays noncompetitive inhibition kinetics against BoNT/A LC [133].

Despite intensive efforts on BoNT inhibitor development, to date, no compounds have yet been identified that exhibit suitable characteristics (safety, efficacy, solubility) and possess ADMET (absorption, distribution, metabolism, excretion and toxicity) profiles to make them effective pharmaceutical interventions. Part of the extreme difficulty in the BoNT/A inhibitor development can be attributed to the unusually large peptide substrate-enzyme interface [131] that requires a small-molecule with high affinity to effectively disrupt protein-protein interactions, i.e., block substrate binding [126]. Moreover, the BoNT toxin and its domains show considerable conformational flexibility, making design of effective inhibitors complicated [128].

Assays and model development are also critical to the BoNT therapeutic effort. A number of in vitro, cell-based, tissue-based, and in vivo assays have been developed (reviewed in Hakami et al. [144]). Though advances in the development of high throughput screening and cell-free biochemical assays have greatly enhanced the initial identification and testing of candidate BoNT inhibitors, the non-correlation between in vitro and in vivo results [127, 145] emphasized the limitations of conventional drug development archetypes. Greater research is vital toward the development of in vitro assays that will allow prompt identification of BoNT inhibitors that are effective both in vitro and in vivo. An added challenge is the demonstration of the in vivo efficacy of candidate small molecule inhibitors. Targeting of drugs to the nerve terminal, ensuring their access to the intracellular compartments and increasing their bioavailability to match the duration of the toxin will be aided enormously by the efforts and progress on delivery vehicle research.

Summary

Botulinum neurotoxins are extremely potent toxins produced by a number of clostridial species that are found worldwide. Their diversity poses challenges for detection/diagnosis, treatment, and countermeasures. Deliberate introduction of BoNTs may be hazardous to both humans and domestic animals, and could cause widespread economic damage.

Recent developments in detection have produced extremely sensitive and specific rapid assays that detect and serotype BoNTs, particularly those known to be human pathogens. In addition, strain- or isolate-level identifications are now possible.

New antitoxin treatments have been developed. A new equine heptavalent antitoxin has been approved for use in adults, and a human antibody product is available for the treatment of infant botulism. Recombinant human-human- compatible antibodies are being produced that effectively neutralize a range of BoNT serotypes and subtypes. Research to develop drug therapies is also underway.
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