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Rational Design of Therapeutic and Diagnostic against Botulinum Neurotoxin

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Abstract

In September 2006, several cases of botulinum poisoning were reported in United States and Canada due to consumption of commercial organic carrot juice. This incident led to the hospitalization of several individuals who received intensive ventilator support. In spite of botulinum neurotoxin being the most poisonous material, little is known about its mechanism of binding, effective drugs are lacking, and correct diagnosis of botulinum poisoning is slow. Fast and accurate diagnosis of botulinum poisoning (through protein fingerprinting), and new rational drug designs are needed to supplement the current protocol for treatment of botulinum poisoning: administration of antitoxin, adequate mechanical ventilation, and meticulous and intensive care. This technical memorandum reviews past and present research & development efforts on botulinum neurotoxins and future pre-clinical drug discovery directions at DRDC Suffield. A comprehensive drug discovery process is described, including high throughput screening FRET assay, rapid and efficient CE-LIF enzymatic assay, equilibrium binding μ-affinity MS assay, cell-based assay, and *in vivo* mouse bioassay. Mechanistically-novel additions to current therapeutics could be in the form of a combination of antitoxin and drugs against cell binding and/or the proteolytic activity of botulinum neurotoxin.

Résumé

En septembre 2006, plusieurs cas d'empoisonnement botulique ont été relatés au États-Unis et au Canada dû à la consommation de jus de carottes organique vendu sur le marché. Cet incident a amené à l'hospitalisation en soins intensifs sous ventilateur de plusieurs individus. La neurotoxine botulique est le poison le plus puissant pourtant on connaît peu son mécanisme de liaison; on manque de médicaments efficaces et le diagnostic correct d'empoisonnement botulique est lent. Il faudrait un diagnostic rapide et exact d'empoisonnement botulique (par cartographie peptidique de protéines) et de nouveaux concepts rationnels de drogues pour supplémenter le protocole actuel du traitement d'empoisonnement botulique: administration d'antitoxine, ventilation mécanique adéquate et soins intensifs méticuleux. Ce document technique étudie la recherche & développement présents et passés, à RDDC Suffield, sur les efforts de mise au point concernant les neurotoxines botuliques et vers quelles directions se dirigent les futures découvertes de médicaments précliniques. On y décrit un processus complet de découverte de médicaments, dont les bio-tests FRET de criblage à haut débit, les bio-tests CE-LIF enzymatiques rapides et efficaces, les bio-tests SM d'affinité μ de fixation en équilibre, les bio-tests à base de cellules et les bio-essais sur souris *in vivo*. Des additions nouvelles aux thérapeutiques actuelles, au point de vue mécaniste, pourraient se présenter sous la forme d'une combinaison d'antitoxines et de médicaments contre la fixation des cellules et/ou contre l'activité protéolytique de la neurotoxine botulique.

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Executive summary

Introduction: Botulinum neurotoxin has been investigated as a possible agent for offensive or retaliatory use by a number of countries in the past, including Canada, due to its extremely high toxicity and ready producibility. It might well be employed asymmetrically by countries of concern or by terrorist groups in the future. In addition, botulism poisoning is an ongoing public health concern. Notwithstanding these concerns, therapy for botulism poisoning has advanced little in the past fifty years. Administration of antitoxin prevents progression of neurologic symptoms, but patients may nonetheless require long term intensive care (including mechanical ventilation). This publication examines possible improvements in therapeutics which could be achieved through better understanding of mechanism of recognition of botulinum neurotoxin (BoNT) to nerve cells, development of an effective drug candidate through a comprehensive drug discovery process, and rapid and mistake-free diagnosis.

Results: The most attractive therapeutic targets are: 1) neutralizing toxin in the blood stream, 2) blocking cellular recognition of BoNT onto cell surface receptor of nerve cells, and 3) blocking/reversing paralysis by inhibiting enzymatic activity in the cytoplasm of the nerve cell. Large collections of peptide compounds were used for screening against BoNT utilizing two functional enzymatic assays. The results showed these peptide libraries were useful as inhibitors and could be used to design small molecule (non-peptide) drugs. A high throughput homogeneous assay and an affinity binding assay are currently being developed to rapidly screen large numbers of peptide libraries and small molecules, and for further deconvolution of the libraries. A cell-based assay is being developed to evaluate the ability of an inhibitor to cross the cell membrane into the cytoplasm where the proteolysis occurs. A rapid detection method based on protein fingerprinting is also being developed.

Significance: The use of a rational design approach to countermeasures against botulinum neurotoxin should allow the development of effective therapeutics (through selection of drug candidates specifically designed against botulism), as well as improved detection. The combination of high throughput screening assay, rapid and efficient enzymatic assay, equilibrium affinity binding assay, cell-based assay, and *in vivo* mouse neutralization bioassay, together with new abilities to understand toxin binding and new strategies in drug designs, will provide a comprehensive capability in the Toxin Research Hub at DRDC Suffield. This capability could be applied to other toxins of concern, such as ricin and tetanus, as well as those produced by infectious agents, such as anthrax.

Future Research: Future R&D efforts will focus on the acquisition of additional mechanistic information and mode of inhibition of each drug candidate, the development of an appropriate animal model for assessment of the efficacy and toxicology of drug candidates, and the determination of cell surface receptor(s) and the peptide sequence on BoNT responsible for the binding with these receptors.

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Sommaire

Introduction: Un certain nombre de pays dont le Canada a étudié la neurotoxine botulique comme agent potentiel du fait qu'elle a été autrefois utilisée comme moyen offensif et de représailles dû à son extrême haute toxicité et à la facilité de sa production. Il se peut très bien qu'elle soit employée asymétriquement, à l'avenir, par les pays qui nous préoccupent ou par des groupes de terroristes. De plus, l'empoisonnement botulique est une préoccupation continue de la santé publique. Malgré ces préoccupations, la thérapie contre l'empoisonnement botulique a très peu avancé depuis cinquante ans. L'administration d'antitoxines prévient la progression de symptômes neurologiques mais certains patients peuvent néanmoins exiger des soins intensifs à long terme (dont la ventilation mécanique). Cette publication examine les améliorations aux thérapeutiques possibles qui permettraient une meilleure compréhension du mécanisme de reconnaissance des neurotoxines botuliques (BoNT) parmi les neurones, la mise au point d'un médicament candidat efficace au moyen d'un processus compréhensif de découverte de médicaments et d'un diagnostic rapide qui ne commettrait pas d'erreur.

Résultats: Les cibles thérapeutiques attirantes sont: 1) la neutralisation de la toxine dans le courant sanguin, 2) le blocage de la reconnaissance cellulaire du BoNT sur le récepteur de la surface de la cellule des neurones et 3) le blocage ou le renversement de la paralysie qui inhibe l'activité enzymatique dans le cytoplasme du neurone. On a utilisé des grandes collections de composés de peptides pour cribler contre le BoNt en utilisant deux bio-tests enzymatiques fonctionnels. Les résultats indiquent que les bibliothèques de peptides ont été utiles comme inhibiteurs et pourraient être utilisées pour concevoir des médicaments de petites molécules (non peptide). Un bio-test homogène ayant un haut débit et un bio-test d'affinité de fixation sont actuellement en voie de mise au point pour cribler rapidement un grand nombre de bibliothèques de peptides et de petites molécules ainsi que pour produire une déconvolution supplémentaire des bibliothèques. Un bio-test à base de cellules est en voie de mise au point visant à évaluer la capacité d'un inhibiteur à traverser les membranes de cellules dans le cytoplasme où la protéolyse se produit. Une méthode de détection rapide basée sur la cartographie peptidique de protéines est aussi en voie de mise au point.

Portée des résultats: L'utilisation d'une méthode conceptuelle rationnelle contre la neurotoxine botulique devrait permettre la mise au point de thérapeutiques efficaces (au moyen de la sélection de médicaments candidats spécialement conçus contre le botulisme) ainsi qu'une amélioration de la détection. Une combinaison de bio-tests de criblage à haut débit, de bio-tests enzymatiques rapides et efficaces, de bio-tests d'affinité de fixation en équilibre, de bio-tests à base de cellules et de bio-essais de neutralisation sur souris *in vivo*, ensemble avec une nouvelle habileté à comprendre la fixation de toxines ainsi que les nouvelles stratégies de conception de médicaments fourniront des capacités compréhensives au centre de recherche sur les toxines de RDDC Suffield. Cette capacité pourrait être appliquée à d'autres toxines inquiétantes telles que le ricin et le tétanos ainsi qu'à celles produites par des agents infectieux tels qu'anthrax.

La recherche future: Les efforts futurs en R & D seront axés sur l'acquisition additionnelle d'information mécaniste et les modes d'inhibition de chaque médicament candidat, sur la mise au point d'un modèle animal approprié à l'évaluation de l'efficacité et de la toxicologie des médicaments candidats et sur la détermination de récepteurs de surface de cellules et de la séquence peptide sur le BoNT responsable de la fixation sur ces récepteurs.

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Introduction

Botulinum neurotoxin (BoNT), of which there are seven serotypes (designated A through G), is the most toxic known substance because of the low dosage required for toxicity (intravenous LD_{50} is 1 ng/kg) [1]. The toxin is known as the cause of botulism, a type of deadly food poisoning produced by improper canning. The extreme toxic nature makes it one of the prime potential biological agents. There is currently no effective cure or treatment of botulism nor is there an effective treatment after exposure. Antitoxin (antibody) therapy has been shown to stop progression of neurologic symptoms but patients remained hospitalized and on ventilators, as seen in the recent occurrence of botulism in North America in September 2006 [2]. Furthermore, there is a lack of general knowledge in the mechanisms of action for toxicity and specifically the essential BoNT domains involved in each process do not allow the rational design of inhibitors. This technical memorandum is a guide to previous and current efforts, and future plans for detection & identification and therapeutics against BoNT at DRDC Suffield.

In the past 20 years, many of the cosmetic and therapeutic benefits of BoNT were discovered and used for wrinkle removal, and diseases such as muscular spasms, neurological and endocrine disorders, including treatment against excess sweating and migraine [3-5]. BoNTs are produced by an endospore-forming, anaerobic, Gram-positive bacilli bacterium, *Clostridium botulinum*, which can be found worldwide in soil. *Clostridium tetani* is a similar bacterium to *C. botulinum*, but produces the tetanus toxin that causes tetanus in humans through cuts and wounds resulting in severe muscle spasms. With botulism, paralysis begins with the cranial nerves, followed by effects to the upper extremities, the respiratory muscles, and finally, the lower extremities in a proximal-to-distal pattern. In severe cases, extensive voluntary muscle paralysis leads to respiratory failure and death unless supportive care is provided [6]. According to the poison information monograph published by the World Health Organization (WHO) in 1999, there are five clinical categories of botulism: 1) foodborne botulism; 2) wound botulism; 3) infant botulism; 4) adult infectious botulism; 5) inadvertent, following botulinum toxin injection [6]. The WHO article was revised in 2002 to include inhalation botulism [7]. The latter form has occurred in laboratory workers following inhalation of the toxin (in an aerosol). In the inhalation cases, neurological symptoms may be the same as in foodborne botulism, but the incubation period might be longer.

Antitoxin against botulism is available in the form of passive immunologicals, such as the CDC (Centers for Disease Control and Prevention) approved equine-derived antitoxin [8] and the human compatible immunoglobulin [9]. However, treatment is not without risk, as approximately 9% of persons treated experience hypersensitivity reactions [9, 10]. Vaccination of the general public is unwarranted due to the low incidence rate of botulism poisoning. Although synthetic inhibitors (small molecule drugs) that target circulating BoNT have the potential advantage of being cheaper to produce than antibody-based therapies, there are currently no drugs under development. A high throughput screening, medicinal chemistry structure-based iterative process along with analytical chemistry and pharmacology assays are needed for drug discovery, and subsequent cell and animal studies are essential to develop effective therapeutics. The most attractive therapeutic targets are 1) neutralizing toxin in the blood stream, 2) blocking cellular recognition of BoNT onto cell surface receptor of nerve cells, and 3) blocking/reversing paralysis by inhibiting enzymatic activity within the cytoplasm of the nerve cell.

The passive immunotherapies are thought to be effective only in binding and neutralizing BoNT that are still in the blood stream prior to cellular recognition, and thus their usefulness is limited to a narrow window of time post-exposure. Current research and development (R&D) of BoNT countermeasures are based on developing inhibitors against the enzymatic activity of the toxin and can be assayed by traditional methods. However, inhibitors against the enzyme activity, by definition, can only be effective after the toxin has entered the cell and thus clinicians are left with a relatively short therapeutic window to neutralize the toxic effects. Additionally, inhibitors are peptide-based, thus researchers have to face the problem of low bioavailability due to metabolic breakdowns and difficulty in crossing cell membranes. An addition to current strategy would be to develop inhibitors that prevent BoNT from entering the nerve cells, a process regulated by the heavy chain of BoNT. This is a challenging task as limited information is known about the mechanism of heavy chain binding, furthermore the heavy chain cannot be assayed via traditional function-based assays. Moreover, there are still considerable gaps in our knowledge of the biology of the mechanisms of transport from the lumen of the gastrointestinal tract or lung into the plasma, endocytosis into peripheral cholinergic nerve cells, translocation to the cytoplasm of the nerve cell, substrate recognition domains and persistence within the cytoplasm.

In the past few years, BoNT research and development (R&D) efforts at DRDC Suffield has been focused on combinatorial peptide libraries as potential inhibitors. These peptide libraries formed the basis to obtain structural and conformational information in order to design better small molecule drugs against BoNT [11-14]. Research was done primarily with assays to study the effects of combinatorial peptides (typically tetra- or tri-peptide molecules) on BoNT/A and BoNT/B. Much analytical work was developed to separate and quantitate the peptide substrates and enzymatic products to determine the modulating effects of peptide libraries. The enzymatic assays were followed by either HPLC separation with UV detection [15], or by capillary electrophoresis (CE) separation with UV or LIF (laser-induced fluorescence) detection [16]. The purity and integrity of selected peptides were analysed by mass spectrometry. These peptide libraries were prepared in a synthetic combinatorial library fashion to produce large numbers of peptide products for high throughput screening. These peptide libraries offer molecular and conformational diversities. The peptides have the structures of acetyl-X1-X2-GABA-X3-X4-NH_2, where X1 to X4 are near-equimolar mixtures of 12 amino acids and GABA is γ-aminobutyric acid, which is an uncommon amino acid with a more flexible backbone than all other common α-amino acids (Fig. 1). The N-, and C-termini of all peptides are acetylated (Ac-) and amidated (-NH_2) respectively. Visually, Table 1 shows the distinct differences in functionality of the restricted 12 (out of 20) amino acids.

Current BoNT R&D efforts are focused on rapid detection of BoNT in the development of a peptide fingerprint by capillary electrophoresis, R&D of countermeasures against BoNT light chain (LC) with small molecule drug discovery with fluorescence resonance energy transfer (FRET) and cell-based assays. Future efforts will focus on detail studies of small molecule inhibitors with enzyme kinetics and binding affinity determinations, as well as animal studies of drug candidate(s) to develop therapeutics against the LC of BoNT. Additionally, a new approach to botulism countermeasure will start by determining the site(s) of receptor binding of the BoNT heavy chain, targeting those binding sites and designing antagonists against these sites, and thus blocking cellular recognition of BoNT onto cell surface receptor of nerve cells. This new approach has the potential in preventing BoNT from entering nerve cells and resulting in neurological damages and flaccid paralysis.

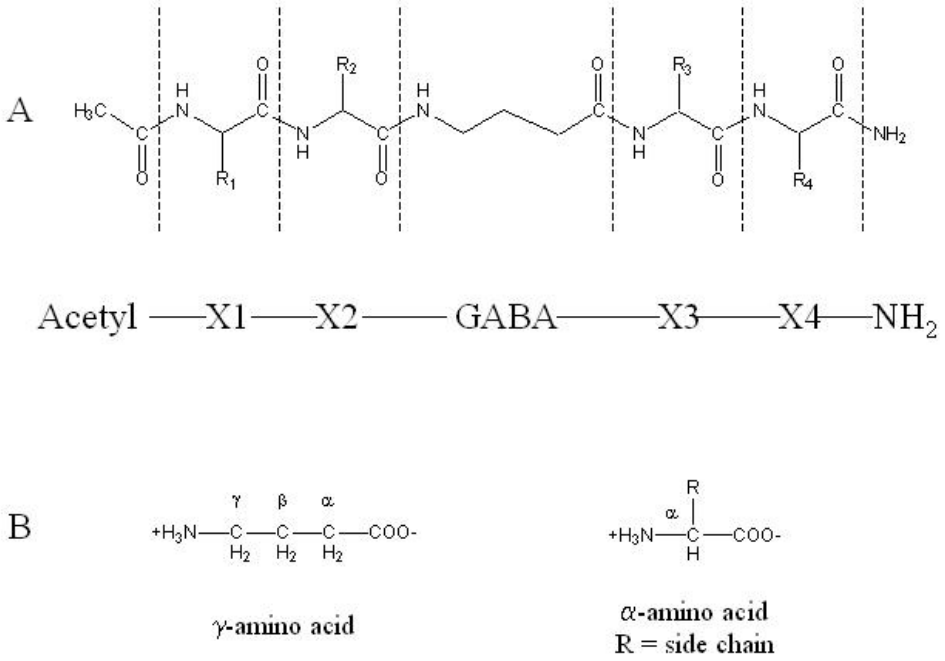
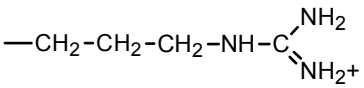


Figure 1. A, General structure of a tetrapeptide with GABA. The N-terminal is acetylated, and the C-terminal is amidated, while X1 through X4 are any one of 12 amino acid residues selected from Table 1. B, Comparison of GABA (γ-aminobutyric acid) to an α-amino acid shows the three carbon backbone of GABA provides large range of flexibility to the peptide for conformational diversity.

Table 1. Basic Characteristics of the 12 α-amino acids chosen for use in combinatorial peptide libraries

Amino acids (X)	1-letter symbol	Side chain structural formula	pKa side chain
Arginine	R		12.48
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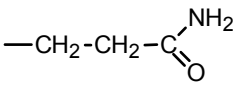
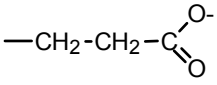
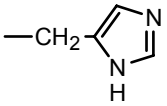
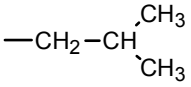
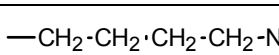
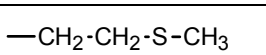
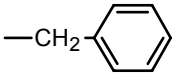
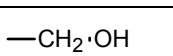
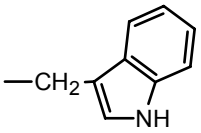
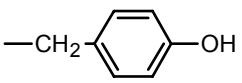
Glutamine	Q		-
Glutamic acid	E		4.07
Histidine	H		6.04
Leucine	L		-
Lysine	K		10.79
Methionine	M		-
Phenylalaine	F		-
Serine	S		-
Tryptophan	W		-
Tyrosine	Y		10.13

Overview of botulinum neurotoxins

Botulinum neurotoxin is a protein complex consisting of a light chain (LC, 50 kDa) and a heavy chain (HC, 100 kDa), covalently linked together by a single disulfide bond [17]. The HC is responsible for irreversible binding to the cell surface receptor on the nerve cell prior to entering through endocytosis. The light chain (LC) is a Zn-dependent endopeptidase [18-21]. The effect of BoNT at presynaptic neuromuscular junction terminals is to cleave the proteins involved in the transport of acetylcholine containing vesicles, thereby preventing the release of neurotransmitter, acetylcholine [22, 23]. BoNT blocks the autonomic postganglionic terminals from releasing acetylcholine resulting in muscle paralysis. Figure 2 shows a comparison of the normal event of SNARE (**S**oluble **N**SF (*N*-ethylmaleimide-sensitive fusion protein)-**a**ttachment protein **re**ceptor) protein complex formed and release of neurotransmitter, and the event disrupted by BoNT cleaving the SNARE proteins.

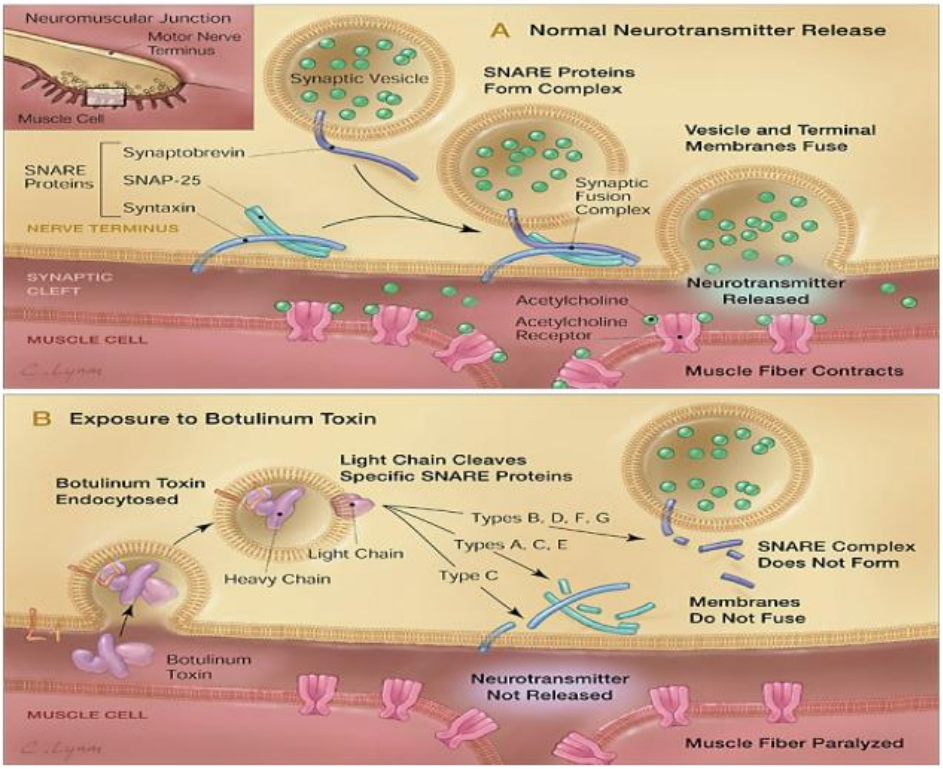


Figure 2. Mechanism of action of botulinum neurotoxin. A: Release of acetylcholine (green dots) at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25, and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell. B: Botulinum neurotoxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum neurotoxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum neurotoxins serotypes B, D, F and G cleave synaptobrevin; serotypes A, C, and E cleave SNAP-25; and serotype C cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract. (after [24])

There are three stages of mechanism of action for toxicity: binding, internalization, and enzymatic activity. The mechanism of action [25-27] of each of the seven serotypes is similar. The stages are depicted in Fig. 2B. In the first stage, a domain at the carboxyl end of the holotoxin (full length toxin of LC and HC connected by a disulfide bond) recognizes and binds to a receptor on the surface of the target neuron [28]. The internalization and translocation stage follows, which results in delivery of the LC of the toxin into the cytoplasm. When the LC gains entry into the cytoplasm, it is still unclear whether all or part of the HC also enters the cytoplasm. Finally, once in the cytoplasm, the LC behaves like an endopeptidase and cleaves the corresponding substrate, preventing the acetylcholine-containing vesicle from fusing to the membrane and subsequently preventing acetylcholine release into the synaptic cleft [9, 17, 21, 24, 29-30]. General lack of knowledge of these mechanisms (binding sites on neuronal cells, endocytosis and translocation) and specifically the essential BoNT domains involved in each process hinders rational design of inhibitors.

There are seven serotypes (distinctive antigenic properties) of BoNT, designated A through G. Interestingly, not all serotypes have been associated with human poisoning. Serotypes A, B, E, and F have been identified in numerous human poisoning episodes [9]. Each of the seven serotypes will cleave one of the three SNARE proteins at specific sites (Fig. 3): BoNT/A, C, E cleave SNAP-25 (Synaptosomal-associated protein of 25 kDa), BoNT/B, D, F, and G cleave synaptobrevin, and BoNT C cleaves syntaxin. SNAP-25, synaptobrevin and syntaxin form the SNARE protein complex which mediates the fusion of synaptic vesicles with the neuronal membrane. Acetylcholine is released into the synaptic cleft and then binds to acetylcholine receptors on muscle cells for normal nerve impulse and muscle contraction. Cleavage of the neuronal SNARE complex proteins by BoNTs disrupts the synaptic vesicle fusion and prevents acetylcholine release.

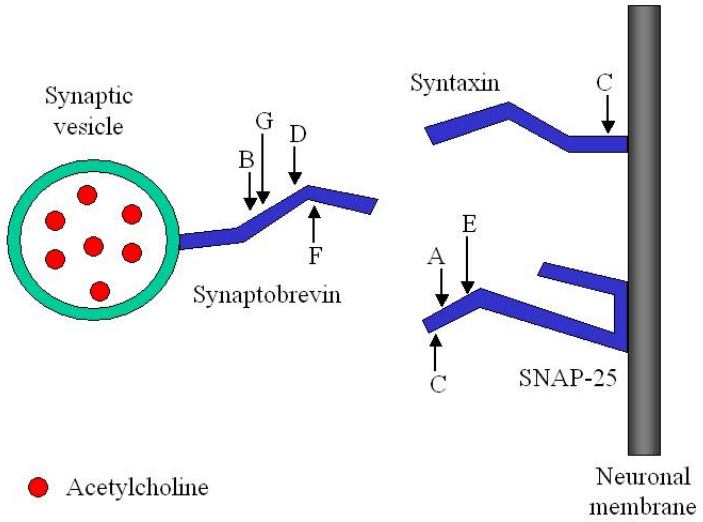


Figure 3. Cleavage sites of each botulinum neurotoxin on the SNARE protein complex. Synaptobrevin on the synaptic vesicle, containing acetylcholine (red circles), must interact with syntaxin and SNAP-25 on the neuronal membrane for synaptic fusion to occur, which allows the nerve impulse to be delivered across the synaptic junction. The botulinum neurotoxin serotypes cleave the peptide bonds at specific sites on the three proteins, as indicated by the arrows and the alphabetic labels. Cleavage of any one of these proteins prevents vesicle membrane docking and nerve impulse transmission.

Previous R&D efforts :

HPLC/UV inhibition assay of combinatorial peptide libraries

In 2000, Defence Research Establishment Suffield (currently DRDC Suffield) started a Technology Investment Fund (TIF) project based on rational drug design of therapeutic peptides to treat and detect & identify toxin threats. The approach was to develop focused mini-libraries of peptides based on combinatorial library chemical synthesis to screen for small molecule compounds that would have the most specific or potent effects against toxins of interest, namely BoNT/A, BoNT/B, and saxitoxin. The purpose was to find peptides that would inhibit the enzymatic domain of BoNT/A, and BoNT/B, and act as antagonist against saxitoxin.

The rational design of a focused library of combinatorial peptides is based on the following criteria. 1) Use of peptide libraries will circumvent the requirements of iterative screening of large numbers of compounds individually (a tetrapeptide library contains 160,000 (20^4) different peptides). 2) Small peptides of only three or four amino acid sidechains were found to be involved in forming clusters with a pharmacophoric pattern [31]. 3) a flexible central linker embedded in the middle of the peptide sequence allows for disruption of the ordered peptide backbone structure and flanking amino acid sidechains cluster together. 4) Elimination of functional group redundancy in amino acid sidechains (eg. D/E, N/Q, S/T), and structural amino acids that have little functional role (G, P, A, I, V) resulting in further reduction in the library size (20,736 (12^4) peptides). These criteria effectively allow for an 85% decrease in the size of the combinatorial libraries without loss of efficiency, chemical diversity, and concomitant decrease in iterative screening and consumption of reagents and labour.

The first set of experiments [32] was done with a synthetic 17-mer peptide substrate (Ac-SNKTRIDQAN**QR**ATKML-NH_2), which was derived from the C-terminal of SNAP-25 (residues 187 – 203) containing the BoNT/A targeted scissile **Q-R** peptide bond [33, 34]. The N-, and C-termini of all peptides were acetylated (Ac-) and amidated (-NH_2) respectively. BoNT/A was activated in the presence of dithiothreitol (DTT) to reduce the disulfide bond and release the LC (enzymatic domain). The substrate was incubated with BoNT/A LC in a buffer containing Zn^{2+} and DTT, and in the absence or presence of a peptide library. After the incubation, the reaction mixtures (substrate and products) were separated by reversed-phase high performance liquid chromatography (RP-HPLC) for quantification of each component to calculate % inhibition (Figure 4). The peptide libraries were synthesized based on the preponderance of acidic residues in the SNARE motif (repeating sequences in all three SNARE proteins) that were found to inhibit the actions of BoNTs [35]. Thus, the first set of peptide library has the structure of Fig. 1A, where X1, X2, X3, and X4 are equimolar mixtures of D, E, Q, R (acidic residues and Q & R represent residues recognized by BoNT/A LC) [32].

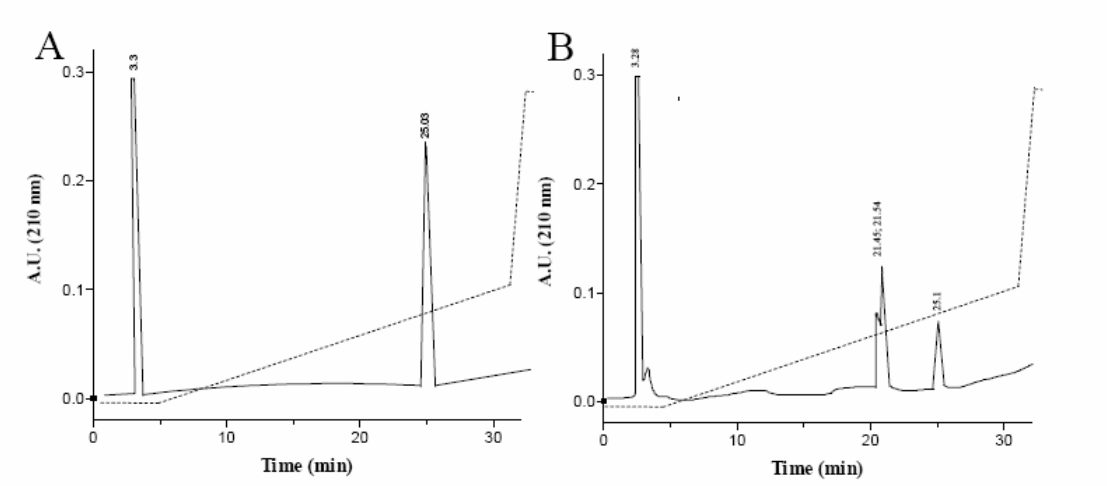


Figure 4. Reversed-phase high pressure liquid chromatography of the synthetic substrate Ac-SNKTRIDQANQRATKML-NH_2 before (A) and after (B) treatment with BoNT/A; substrate elutes at 25 min, and products co-elute at 21.45 and 21.54 min. HPLC was run at a flow rate of 1 mL/min with a column temperature at 37°C with a BioRad Hi-Pore reverse phase 25 cm x 4.6 mm column. The gradient elution started with 100% eluent A (0.1% TFA/H_2O), then a linear gradient to 45% eluent B (0.1% TFA/ H_2O / 70% acetonitrile), followed by a rapid increase to 100% eluent B (see dotted line). (after [32])

Further studies, under the same assay conditions described above, showed zinc-chelating compounds had strong inhibition effects on BoNT/A [11]. Several thio-containing compounds, such as DTT, potent chelators of heavy metal (2, 3-dimercaptopropanesulfonate (DMPS), mercaptosuccinic acid (MSA)) [36-40], and captopril (known Zn peptidase inhibitor [41]) were tested along with peptide libraries with acidic residues (D, E) and thio-containing residues (C, H). One peptide library (256 peptides) containing D, E, C, H residues showed more than 50% inhibition against BoNT/A at 0.5 mM total concentration. Captopril showed 76% inhibition against BoNT/A at 1 mM [11].

A large scale screening effort followed with a full set of peptide libraries in the form of:

Ac-X1-X2-GABA-X3-X4-NH_2 (capped) or X1-GABA-X2-X3 (uncapped)

These had the same definition as given on page 2, except uncapped libraries had peptides possessing free amine and carboxylate at the N- and C-termini respectively in order to reduce synthetic steps [15]. The *in vitro* assay conditions for BoNT/A were similar to that described above. The *in vitro* assay conditions for BoNT/B required activation by DTT, followed by incubation with a 35-mer synthetic substrate representing residues 60-94 of synaptobrevin [42, 43]. HPLC separation conditions were the same for reaction mixtures in each BoNT/A, and BoNT/B assay. The bioassay conditions for saxitoxin inhibition studies were conducted as described by Manger et al. [44]. Briefly, mouse neuroblastoma cells were treated with ouabain, vertatridine and saxitoxin in the absence or presence of peptide libraries. Saxitoxin rescues mouse neuroblastomas from cell death caused by ouabain/vertridine, while the effects of peptide libraries were measured by inhibition of the saxitoxin rescue of ouabain and veratridine toxicity. The results showed modulations (amplification or inhibition) of activities

in all three toxin assays [15]. Strong inhibitions were observed when amino acid residues were fixed at the C-terminal (X4 or X3 in the capped or uncapped libraries, respectively) and on the opposite side of GABA with respect to the C-terminal (i.e. X2 or X1 in the capped and uncapped libraries, respectively). The significant inhibition results of the peptide libraries are summarized in Table 2.

Table 2. Combinatorial peptide libraries that show significant inhibition against BoNT/A, BoNT/B and saxitoxin (after [15])

Capped tetrapeptide libraries Ac-X1-X2-GABA-X3-X4-NH_2		Uncapped tripeptide libraries X1-GABA-X2-X3	
BoNT/A	% Inhibition		% Inhibition
M2C4	75	C1S3	75
S2R4	50	S2R3	51
S2C4	43	M1E3	41
		W1F3	35
BoNT/B			
C2C4	74	C1F3	80
R2R4	60	K1F3	65
F2R4	44	M1F3	48
Saxitoxin			
H2R4	75	E1E3	98
R2R4	65	E2E3	70
		C1F3	42
		C1E3	40

Note. Total peptide library concentrations were 0.5 mM

GABA is γ-aminobutyric acid

1-letter amino acid symbol represents specific residues which were fixed at the indicated positions, i.e. M2C4 is the library Ac-X1-M2-GABA-X3-C4-NH2 with X = near-equimolarity of 12 amino acids, while C and M were substituted at positions 2 and 4.

Though RP-HPLC was used to separate synthetic peptide from the cleaved products, the resolution was poor and was unable to baseline resolve the cleaved products (see Fig. 2B, elution times 21.45 and 21.54 min). This resulted in difficulties with quantifying the amount of each cleaved product produced using area under each peak, thus creating problems in determining inhibitions accurately. In order to achieve better separation and lower consumption of reagents, a method coupling capillary electrophoresis separation and laser-induced fluorescence detection was developed to analyze the assay mixtures.

Capillary electrophoresis inhibition assay of peptide libraries

A fast, efficient capillary electrophoresis laser-induced fluorescence (CE-LIF) method for measuring BoNT/A activity was developed at DRDC Suffield [16]. The conditions for BoNT/A assay were the same as described in the previous section. The enzyme assay products were labelled with 3-(4-carbozybenzoyl)-2-quinoline-carboxaldehyde (CBQCA) dye followed by CE separation and LIF detection in less than 8 minutes with complete resolution of products (P1 and P2) and substrate (S), see Figure 5 (a). Figure 5 shows a typical CE-LIF result of an admixture of CBQCA-labelled substrate (S), and synthesized products, P1 and P2. The 17-mer substrate, having the largest molecular weight (MW 1989 Da) of the mixture and a net +3 charge, migrates to the negatively charged detection end with the faster speed, followed by P2 (MW 719 Da) having a net +3 charge, and finally P1 (MW 1288 Da) having a net zero charge at neutral pH. This CE-LIF method was also used to screen selected peptide libraries. The CE-LIF method improved the enzymatic assay capability with full resolution of product peaks and quantitative measurements by intensity (linear relationship with concentration), and quick analysis time (<8 minutes vs. 60 minutes).

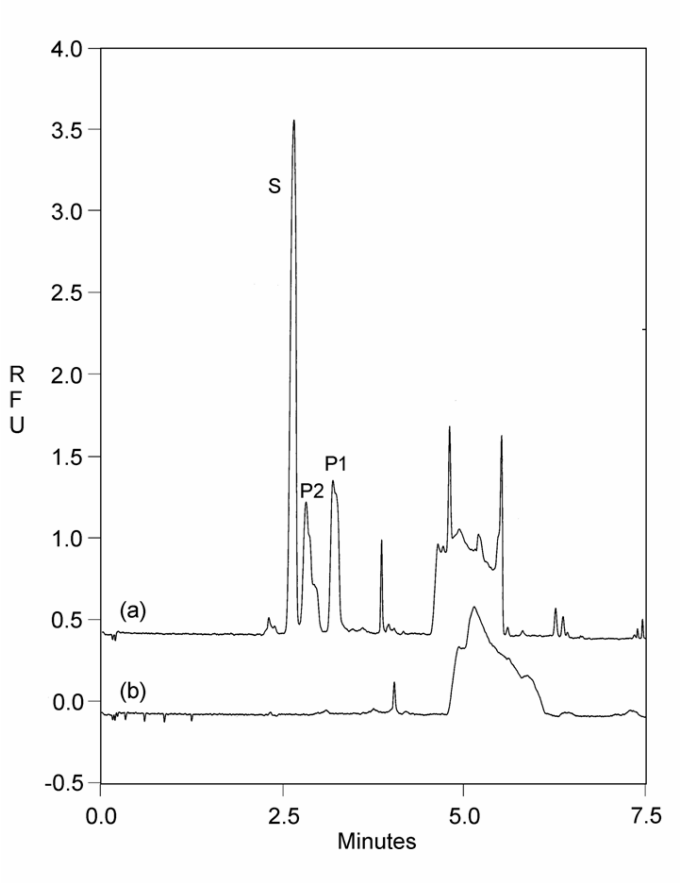


Figure 5. (a) Typical CE-LIF of an admixture of CBQCA-labelled substrate (S), and synthesized products (P1 and P2), and (b) buffer-only control with CBQCA reagent.

Mass spectrometric analysis of selected peptide libraries

Two samples of peptide libraries, Ac-M-GABA-X-C-NH_2 (designated as M1C3) and Ac-H-GABA-X-R-NH_2 (designated as H1R3) were subjected to content analysis by HPLC and mass spectrometry. The M1C3 dry powder was dissolved in 10% acetonitrile/water, then vortexed and ultrasonicated. The final concentration of peptide was approximately 5 mg/mL for injection into a high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) experiment. H1R3 was dissolved in 10% DMSO/ 0.1% trifluoroacetic acid (TFA)/water, resulting in a concentration of approximately 20 mg/mL for HPLC-ESI-MS. A reversed-phase C-18 column was used for the separation of peptides. A linear solvent gradient was used in the HPLC separation at an initial condition of 5% acetonitrile in 0.1% TFA/water, up to 75% acetonitrile/0.1% TFA/water.

HPLC-ESI-MS analysis of M1C3 showed the presence of at least 12 components which matched the monoisotopic molecular masses of the protonated species (see Figures 6-8). LC-ESI-MS analysis of H1R3 showed the presence of nine out of 12 peptides in the library (data not shown). Figure 6 shows two ion chromatograms of M1C3 library. The lower panel shows the total ion chromatogram (TIC, ion effluent from the C-18 column as a function of time). The upper panel shows the extract ion chromatogram (EIC) of m/z between 200 and 700 amu. Ions outside of the mass range do not correspond to the peptides in the M1C3 library. The difference between TIC and EIC is the additional broad band from 1.9 to 2.5 min in TIC corresponding to the solvent, acetonitrile. A collection of EICs showing peaks reflecting each peptide in the library was generated by selecting the specific monoisotopic molecular mass of the peptide. Figure 7 & 8 show the EIC peak collection against the TIC.

The MS results indicated that most of the nominal constituents of the peptide libraries were represented but there were some difficulties in obtaining pure and complete peptide preparations in a library format. Further work is required to achieve complete synthesis of peptides in libraries and develop purification procedures to remove impurities.

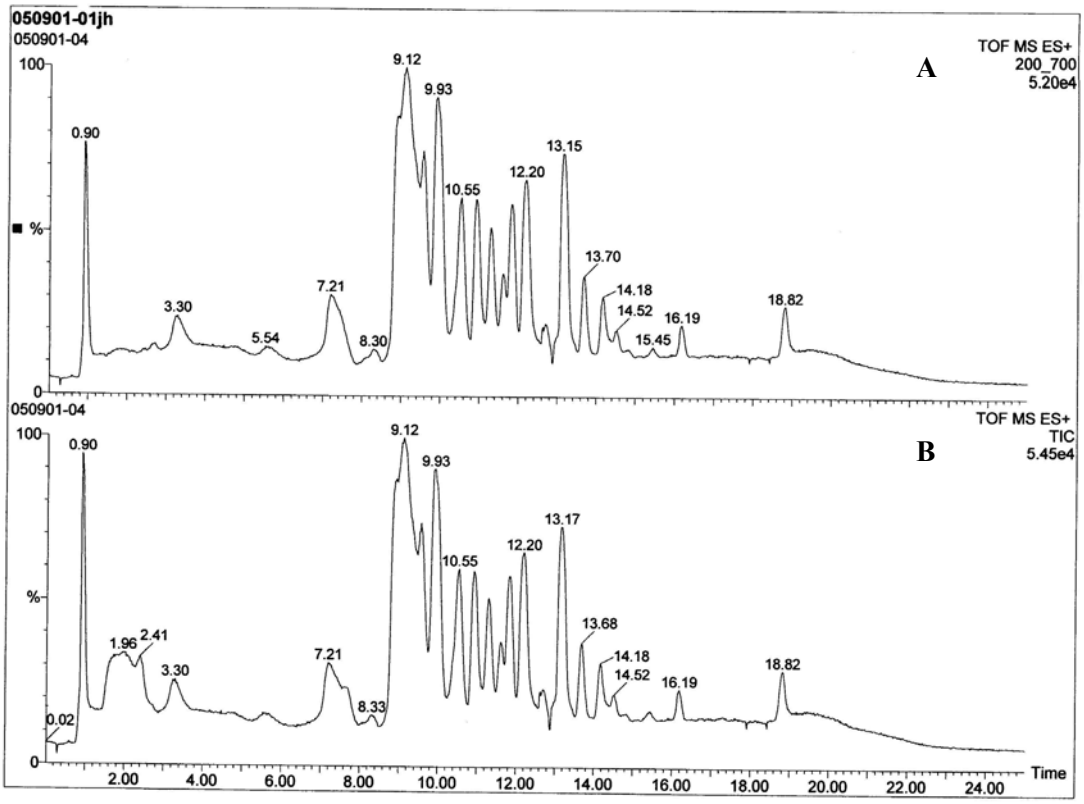


Figure 6. Ion chromatograms of Ac-M-GABA-X-C-NH_2 library. Lower panel shows the total ion chromatogram (TIC) while upper panel shows extracted ion chromatogram (EIC) of m/z between 200-700 amu. TIC shows generally all peaks as seen in the EIC. The additional broad band between 1.9 and 2.5 min corresponds to organic solvent acetonitrile.

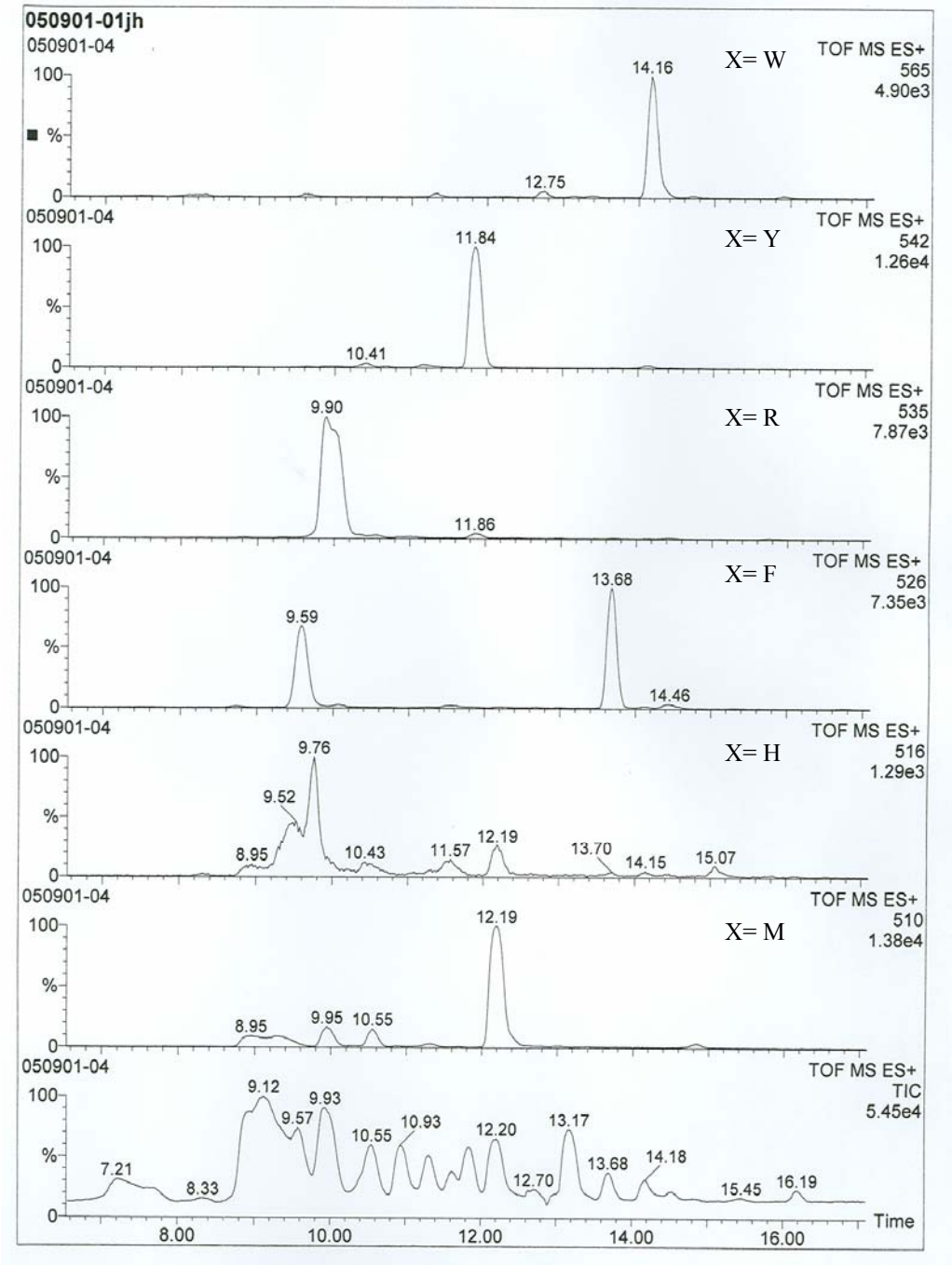


Figure 7. Total ion chromatogram (bottom panel) and each selected ions ascending from selected m/z 510 amu to 565 amu. The library contains Ac-M-GABA-X-C-NH_2, and the amino acid (X) corresponding to each m/z is indicated on each selected chromatogram. This collection of ion chromatograms is generated by selecting each ion (eg. Ac-M-GABA-W-C-NH_2 at 565.22669 amu at the top panel) and the MS software reflects the peak extracted from the ion chromatogram representing that specific ion.

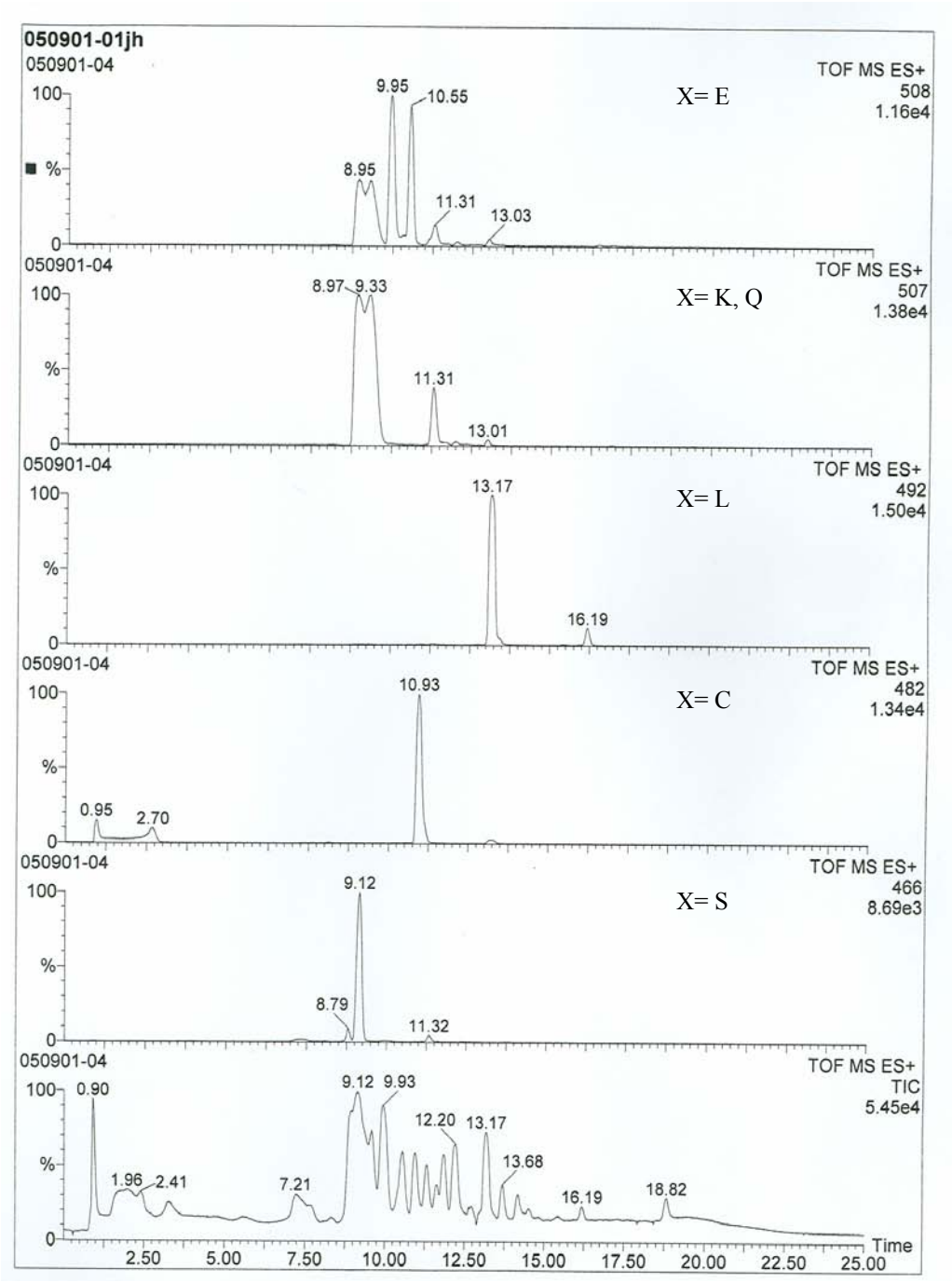


Figure 8. Total ion chromatogram (bottom panel) and each selected ions ascending from selected m/z 466 amu to 508 amu. The library contains Ac-M-GABA-X-C-NH_2, and the amino acid (X) corresponding to each m/z is indicated on each selected chromatogram. This collection of ion chromatograms is generated by selecting each ion (eg. Ac-M-GABA-E-C-NH_2 at 508.18999 amu at the top panel) and the MS software reflects the peak extracted from the ion chromatogram representing that specific ion.

Current R&D efforts :

Protein fingerprinting for rapid detection of BoNTs

The September 2006 occurrence of botulism associated with commercial organic carrot juice in USA and Canada resulted in several people hospitalized on life support ventilators. In a CDC report, suspected cases were first reported on the 8th of September and antitoxin was dispatched that evening, though test results confirming BoNT/A in patients were reported on the 13th of September [2]. CDC recommends "it is therefore extremely important that physicians recognize botulism as early in its course as possible, and yet not mistake other neurologic syndromes for botulism" [9]. A rapid and mistake-free diagnosis of botulism is a must.

Protein fingerprinting for identification of BoNTs based on CE-LIF can be achieved quickly and consume very small amount of sample. The diagnostic sample containing BoNT would be partially purified on an affinity-capture column, and then treated with protease to yield specific peptide fragments. Then these peptides would be chemically modified with a fluorogenic label. CE separation with LIF detection of these peptides can yield the unique pattern of peptides (fingerprint) for detection and differentiation of BoNTs. This new approach can be used as a combination to the current methods of detection: PCR techniques targeting the neurotoxin gene, bacterial cultures, and mouse lethality assay.

High throughput FRET homogenous enzymatic assay

Fluorescence resonance energy transfer (FRET) is the physical process by which energy is transferred non-radiatively from an excited molecular fluorophore (the donor) to another chromophore (the acceptor) by means of intermolecular long range dipole-dipole coupling [45, 46]. The essential requirements for effective transfer over distances from 10 Å to 100 Å between the donor and the acceptor are that their fluorescence spectra overlap, and the quantum yield of the donor and the absorption coefficient of the acceptor are high. This process leads to development of various homogenous enzymatic assays where fluorescence resulting in cleavage of a FRET substrate is monitored and directly correlated to the enzyme activity. Massively parallel FRET enzymatic assays can be done because physical separation of product from substrate is not required, thus it is possible to develop high throughput screening of inhibitors against the enzyme.

SNAPtide is the synthetic peptide substrate specifically designed for BoNT/A LC for use in the FRET assay. SNAPtide contains the sequence spanning the $\mathbf{Q}_{197}$-$\mathbf{R}_{198}$ peptide bond, an N-terminally-linked fluorophore, o-aminobenzoic acid (o-Abz), and an acceptor chromophore, 2,4-dinitrophenol (Dnp). o-Abz/Dnp-SNAPtide (5 μM) was mixed with 10 nM BoNT/A LC in a 20 mM HEPES buffer, pH 8.0 with 0.3 mM $ZnSO_4$, 0.1% Tween 20, and 0.5 mM dithiothreitol (DTT) to a total volume of 100 μL. o-Abz/Dnp-SNAPtide and recombinant BoNT/A are commercially available from List Biological Laboratories, Inc. (San Jose, CA). The reaction mixture was incubated at 37 °C for 30 min, and then stopped with the addition of 400 μL 10 mM Tris, pH 8.0 and 100 mM EDTA. Fluorescence was measured on a

fluorescence spectrophotometer with a 96-well microplate format (Cary Eclipse, Varian Canada Inc., Mississauga, ON) with excitation wavelength at 318 nm, and emission wavelength at 421 nm. This assay system provides a fast evaluation method for testing a new peptide library set, capped tripeptides:

$$Ac\text{-}X1\text{-}GABA\text{-}X2\text{-}X3\text{-}NH_2 \qquad (capped)$$

with the same definitions showed on page 8 of this article. This library contains 1728 (12^3) compounds in collections of 12 compounds per set of samples. This FRET assay will be used as a preliminary step, where each set can be screened for inhibition or determine IC_{50} (concentration of inhibitor that reduces enzyme velocity by half) to identify sample(s) containing potential inhibitors. Then, a "drill-down" process allows for deconvolution of the library sets, and eventually testing individual compounds from the active set.

Figure 9 shows a plot of duplicate dose-response curves of Ac-M-GABA-X-C-NH_2 (M1C3) library set of 12 compounds in the FRET assay. The inhibition of BoNT/A LC showed by the M1C3 library prompted a further deconvolution of the set. Dose-responses of individual compounds from the set were evaluated and are summarized in Table 3.

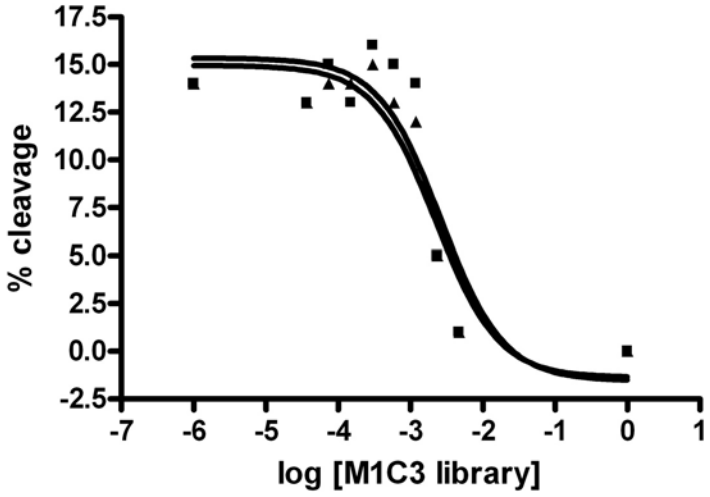


Figure 9. Dose-response curves of a 12-compound mixture of Ac-M-GABA-X-C-NH2 (M1C3) library in the FRET BoNT/A light chain assay. The assay was done in duplicate, represented by squares (■) and triangles (▲).

Table 3. Evaluation of BoNT/A LC inhibition by individual compounds in the Ac-M-GABA-X-C-NH_2 (M1C3) library set

X2 =	BoNT/A LC inhibition?	IC_{50}
R	No	N/A (not available)
C	Yes	568 μM
Q	Undetermined*	2.52 mM
E	Undetermined	1.25 mM
H	No	N/A
L	No	N/A
K	Undetermined	1.08 mM
M	Undetermined	362 μM
F	Weak	3.82 mM
S	No	N/A
W	No	N/A
Y	No	N/A

* BoNT/A LC inhibition was undetermined due to poor non-linear regression fit to the experimental data points, though IC_{50} values were determined, the confidence level is low in the undetermined cases.

Table 3 shows the importance of deconvolution of the library mixture. M1C2C3 was found to be a moderate inhibitor (IC_{50} of 568 μM) among the mixture of 12, while the library mixture (a weak inhibitor) scored an IC_{50} of 2.24 mM. Other peptide libraries were found to be non-inhibitory or possessed very weak inhibition. These screening results are crucial to the drug discovery process for information on inhibitor recognition by the binding site of the BoNT for designing small molecule inhibitors.

Small molecule inhibitor screening

Small molecule (non-peptidic) therapeutics do not exist to counter BoNT poisoning. A combination of small molecule therapeutics and passive immunotherapy could increase the overall effectiveness of treatment against BoNT poisoning. The advantages of small molecules over peptides as inhibitors are: better bioavailability because of presence of proteases in digestive and circulatory systems, and easier transport across cell membrane into the nerve terminal where BoNT LC resides. There are limited reports of small molecule inhibitors of BoNT/A LC by captopril [11, 15], bisquinolines [47], bis-imidazoles [48], and a serotype-selective inhibitor was also identified recently [49].

In our study, a collection of small molecules was selected as potential inhibitors to BoNT/A using virtual screening based on the binding site of reported BoNT/A crystal structure. These small molecules and their structures are presented in Table 4 along with their IC_{50} values determined by FRET assay described previously. The small molecules are presented in ascending order according to their IC_{50} values (i.e. strongest to weakest inhibitor). The capped peptide M1C2C3 was included in at the bottom of Table 4 as comparison to the inhibitory effects of these small molecules.

Table 4. Chemical structures and IC_{50} of selected small molecule inhibitors and M1C2C3 peptide

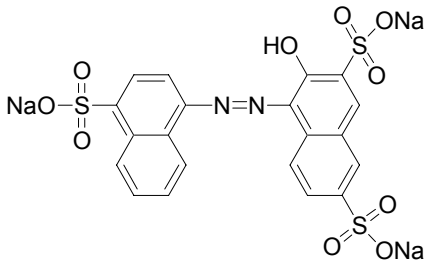
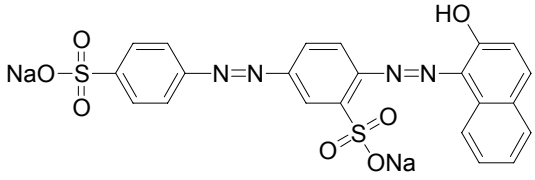
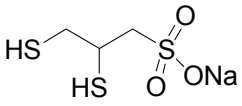
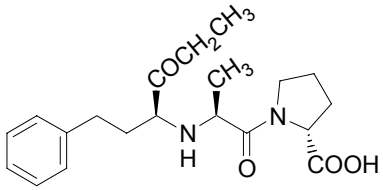
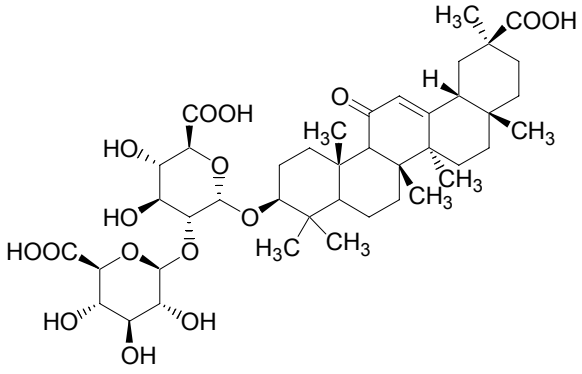
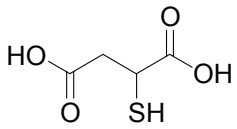
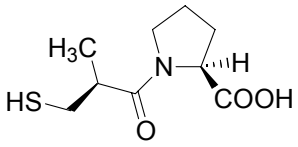
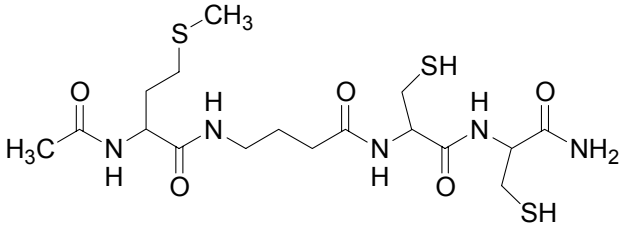
Chemical name	Chemical structure	IC_{50} against BoNT/A
Amaranth		0.11 mM
Ponceau BS		0.13 mM
DMPS		0.58 mM
Enalapril		2.50 mM
Glycyrrhizic acid		2.60 mM

Table 4. Chemical structures and IC_{50} of selected small molecule inhibitors and M1C2C3 peptide (continued)

Chemical name	Chemical structure	IC_{50} against BoNT/A
MSA		4.40 mM
Captopril		5.60 mM
Ac-M-GABA-C-C-NH_2		0.57 mM

DMPS = 2,3-Dimercaptopropanesulfonic acid; MSA = mercaptosuccinic acid; Ac- = acetylated; -NH_2 = amidated

The collection of small molecules selected as potential inhibitors to BoNT/A revealed two relatively strong inhibitors in the sub-millimolar range, amaranth and ponceau BS at 0.11 mM and 0.13 mM respectively. Both compounds are synthetic red dyes: amaranth, also known as FD&C (Food, Drug & Cosmetics) Red No. 2, has been banned by the Food and Drug Administration in the United Stated since 1976 as a suspected carcinogen [50]; ponceau BS, also known as Biebrich scarlet or acid red 66, is a fluorescent marker for basic proteins in cell and tissue staining [51, 52].

The three strongest inhibitors among the small molecules (amaranth, ponceau BS, and 2,3-dimercaptopropanesulfonate (DMPS) with IC_{50} values in the sub-millimolar range) all have at least one sulfonate moiety (SO_3^-). The observation suggested that the more sulfonate moieties, the stronger the inhibitor: amaranth has three sulfonates while DMPS has one, and the IC_{50} values are 0.11 mM and 0.58 mM respectively. The rationale of the observation is not known, but may indicate presence of multiple positive charges on the enzyme active site of BoNT/A. Future work in search for more effective small molecule compounds will have to take this observation into account.

Cell based assay and western blot screening

The aim of the cell based assay and western blot analysis was to verify that 1) SNAP-25 was detectable in cultured neuronal preparations, specifically primary cortical neuronal cultures, routinely prepared in our laboratory and 2) SNAP-25 in the cultured cortical cells was susceptible to specific cleavage by BoNT/A. Once developed, this assay method will be used to screen inhibitors that have been identified from the enzymatic and binding assays.

Briefly, cortical cultures were prepared from 17-19 day old Sprague Dawley rat embryos and grown in neurobasal medium supplemented with B-27 and 0.5 mM glutamine [53]. At 11-14 days in culture, cells were incubated with various concentration of BoNT/A (0, 62.5, 125, 250 or 1000 pM) in the culture media for 24 or 48 h at 37 ºC. Control neurons were maintained in culture medium for 24 to 48 hours. After treatment with BoNT/A, cells were washed with ice-cold PBS and lysed with the following buffer: 20 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl, 1mM EDTA and 0.2% SDS, pH7.5. Proteins (10 µg) were separated by SDS-PAGE on a 12% pre-cast gel (NuPAGE® gel, Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk powder, 0.05% Tween 20 in PBS and then probed with antibodies to SNAP-25 (SNAP-25 monoclonal antibody; Covance SMI-81R, 1:100,000 or Sigma S5187) overnight at 4ºC. After washing, the membranes were incubated with a secondary antibody; anti-mouse IgG-HRP (Amersham Biosciences) diluted 1:10,000. The blots were visualized with ECL advanced detection reagents (Amersham Biosciences) and an imaging system with a CCD camera (VersaDoc; BioRad). Figure 10 shows the C-terminal of the intact SNAP-25 protein (a), and the BoNT/A cleaved C-terminal of SNAP-25(b) and their respective molecular weights.

(a) Intact SNAP-25 [residues 167-206] ~MW

H2N…-MGNEIDTQNRQIDRIMEKADSNKTRIDEAN↓**QR**ATKMLGSG-COOH 25 kDa

(b) BoNT/A cleaved SNAP-25

H2N…-MGNEIDTQNRQIDRIMEKADSNKTRIDEANQ-COOH 24 kDa

Figure 10. C-Terminal sequence of intact SNAP-25 (a) and BoNT/A cleaved SNAP-25 (b). Arrow indicates the BoNT/A cleavage site.

Experimental results showed SNAP-25 was present in primary cortical neuronal cultures, and cleavage of SNAP-25 by BoNT/A was detected by immunoblot. Figure 11 demonstrates proteolysis of SNAP-25 by BoNT/A in rat neuronal cultures but the cleaved product was detected by one of two primary antibodies in Western blot analysis. SNAP-25 protein was evident in the control cell cultures. Incubation of cultures with increasing amount of BoNT/A for 24 h led to an increase in SNAP-25 cleavage (i.e. concentration-dependent), see right panel in Figure 11.

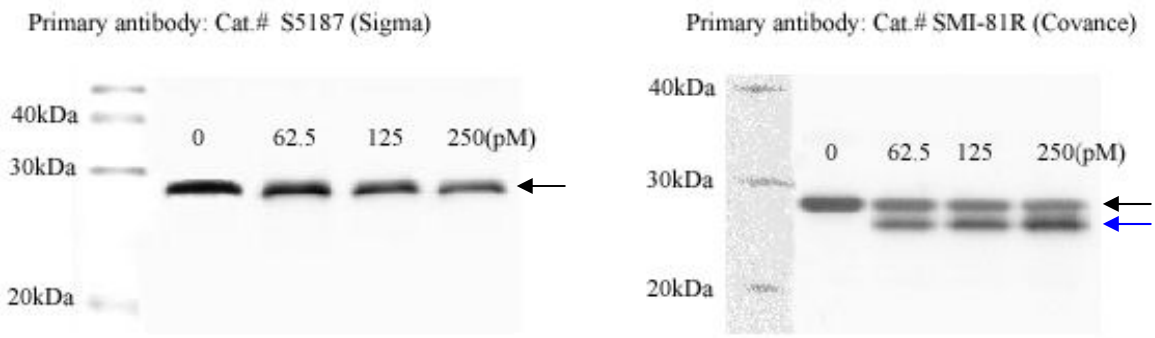


Figure 11. Immunoblot analysis of SNAP-25 in BoNT/A treated neuronal cultures. Cortical neuronal cultures were incubated in various concentrations of BoNT/A (0, 62.5, 125, 250 pM) for 24h. Cell lysates were prepared and analyzed for SNAP-25 immunoreactivity using two different antibodies, S5187 antibody from Sigma (left) and SMI-81R antibody from Covance (right). S5187 antibody did not recognize the cleaved product showing one band representing intact SNAP-25 (black arrow) while SMI-81R bound to both intact and cleaved SNAP-25 (blue, or lower, arrow). BoNT/A cleavage of SNAP-25 was concentration-dependent.

Cleavage of SNAP-25 by various concentration of BoNT/A was examined using the SMI-81R antibody after 24 and 48 hours of toxin exposure and showed time-dependent cleavage (see Figure 12). This result showed concentration- and time-dependent proteolysis.

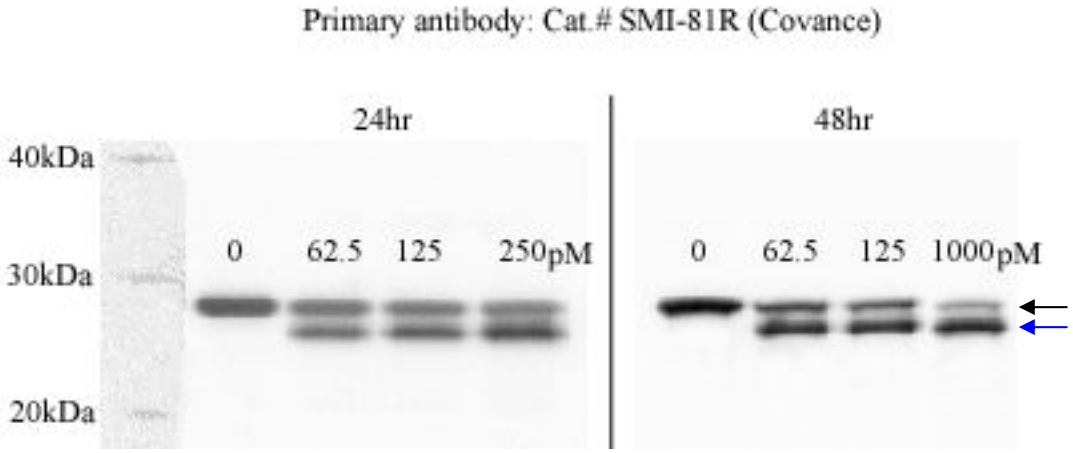
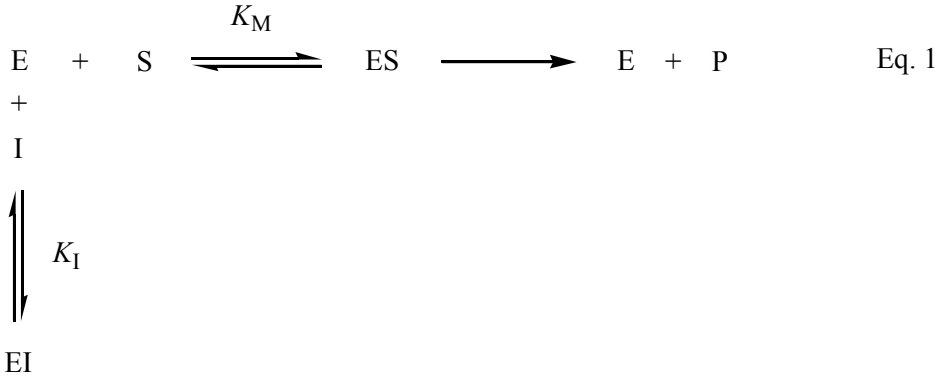


Figure 12. Dose response and time course analyses of SNAP-25 cleavage in BoNT/A treated neuronal cultures. Cortical neuronal cultures were incubated in various concentrations of BoNT/A (0, 62.5, 125, 250 or 1000 Pm) for 24 h or 48 h. With increasing concentration of BoNT/A more of the intact SNAP-25 (black arrow) is cleaved to form the truncated fragment (blue, or lower arrow). At the same concentration, more of the toxin-cleaved SNAP-25 is formed in the 48 h samples.

Future R&D efforts :

Enzyme kinetics of inhibitors

A medicinal enzyme inhibitor is often judged by its specificity (i.e. not binding to other proteins) and its potency (its inhibition constant (K_I), which indicates the concentration needed to inhibit the enzyme). High specificity and potency usually means that a drug will have few side effects and thus low toxicity. To design an inhibitor for BoNT LC thus requires the study of enzyme kinetics to determine the mode of inhibition for rational drug design. Specifically, a competitive inhibitor (I) that binds to enzyme (BoNT LC, E) to form a tight enzyme-inhibitor complex (EI) to prevent BoNT LC from binding to SNAP-25 (substrate, S) is ideal, as shown in equation 1. K_I is the inhibition constant and K_M is the Michaelis-Menten constant.



There are many examples of successful drugs that are competitive inhibitors, methotrexate (an inhibitor of dihydrofolate reductase) and Viagra (an inhibitor of phosphodiesterase [54, 55]) among them. Classifying inhibitors thus needs more than crude measures of IC_{50} values, and if these are used at all they need to be used in conjunction with knowledge of how they relate to inhibition constants (K_I). FRET and CE-LIF assays can be used to determine K_I by titrating different concentration of inhibitor into BoNT/A LC kinetics. These results can lead to further structural improvements to the inhibitor for tighter binding to the enzyme.

Micro-affinity chromatography mass spectrometry binding assay

The understanding of molecular interactions is fundamental to the development of all stages of drug discovery, including study of disease mechanisms, high-throughput screening for the identification of lead compounds, and optimization of lead compounds. Currently, no other alternative method can provide unbiased, direct measures of binding affinity in a rapid and easy to perform manner as micro-affinity chromatography mass spectrometry (μ-affinity MS) [56]. In rational drug design, the aim is to produce an inhibitor with a dissociation constant

(K_d) of $<10^{-9}$ M [57]. This dissociation constant is equivalent to the inhibition constant K_I, though in the absence of substrate.

$$EI \leftrightarrows E + I \qquad \text{where} \quad K_d = \frac{[E][I]}{[EI]} \qquad \text{Eq. 2}$$

One example of the μ-affinity MS method is to screen the peptide library (capped tripeptides, $12^3 = 1728$ compounds) and small molecule inhibitors against a micro-column containing immobilized BoNT/A light chain for molecular binding interaction. This technique allows chromatographic separation based on the strength of the binding interaction between enzyme and inhibitor at equilibrium conditions. Inhibitor breakthrough occurs, at a fixed concentration, when equilibrium is established between inhibitor and immobilized BoNT/A LC. The stronger the binding (lower K_d value), the longer time for inhibitor to reach equilibrium. This longer equilibrium time leads to a longer breakthrough time observed in the chromatogram. A linear direct proportionality exists between the volume of inhibitor solution required to achieve breakthrough ($V_i - V_0$) and the amount of immobilized enzyme (B_t). Thus the lower the capacity of the BoNT/A LC column, the lower the sample consumption.

$$K_d = \frac{B_t}{V_i - V_0} - [A]_0 \qquad \text{or} \qquad V_i - V_0 = \frac{B_t}{K_d + [A]_0} \qquad \text{Eq. 3}$$

Coupling mass spectrometry detection allows additional *m/z* ratio information, making μ-affinity MS amenable to multi-inhibitor binding analyses [58, 59]. A ranking of inhibitor binding strength can be easily obtained in the experimental results. Fitting a non-linear regression curve to data from a titration of an inhibitor at various concentrations ($[A]_0$) through a BoNT/A LC column will simultaneously provide K_d and B_t. Comparing K_d and K_I is important because the two values should be the same if the inhibitor is competitive in nature.

Identification of cell surface receptor for BoNT

Current development of BoNT/A countermeasures are based on developing inhibitors of the light chain as this is the functionally active part of the toxin and can be assayed by enzymatic methods. However, inhibitors against this target, by definition, can only be effective after the toxin has entered the cell and been "activated". A more effective therapeutic strategy would be to develop inhibitors to BoNT entry of nerve cells, a process regulated by the heavy chain of BoNT. However, this is a challenging task as little is known about the mechanism of HC binding, nor can the HC be assayed via enzyme function-based assays. A new approach to BoNT countermeasures will be undertaken, viz., investigation of the mechanism of BoNT HC binding, identification of putative HC receptors, and discovery of small molecules that could potentially interfere with the binding of the HC to the cell surface. Successful completion of these goals will lead to a new strategy for the development of effective countermeasures to botulism poisoning.

It has been proposed that the binding domain of BoNT interacts with two specific components on the cell surface; a ganglioside and a protein receptor [60-62]. Interactions between gangliosides and BoNTs have been reported [63-65] with the exception of BoNT/G [66], and depletion of gangliosides in neuroblastoma cells prevented entry of BoNT/A [67]. Using a combination of site-specific mutagenesis and structural analysis, the sites for cell binding are now known to be located in the C-terminal sub-domain of the HC in BoNT/A and BoNT/B [68].

Ganglioside Binding Site

The requirement for a ganglioside receptor is supported by the fact that BoNT activity is reduced in the presence of lectins [67] and from further binding studies in which it was shown that ganglioside GT1b interacts with the full-length BoNT/A [64]. It has been hypothesized that GT1b causes a change in the HC conformation that permits it to bind or associate with raft-associated protein that internalizes the toxin [69]. Table 3 summarizes gangliosides bound by different serotypes of BoNTs. All references indicated the requirement of sialic acid for BoNT binding, though lactose cerebroside (galatose β1-4 glucose β1-ceramide) will be tested for BoNT binding to compare to all gangliosides.

Table 5. Gangliosides bound by different serotypes of BoNTs (after ref [69])

BoNT serotypes	Ganglioside(s) bound
A	GD1a, GD1b, GT1b, GQ1b
B	GD1a, GD1b, GT1b
C1	GD1a, GD1b, GT1b
D	GT1b
E	GD1a, GD1b, GQ1b
F	GD1a, GD1b, GT1b
G	N/A

N/A = BoNT/G does not require ganglioside binding for entry into target cells [66]

GD1a, GD1b, GT1b, GQ1b are gangliosides with di-, tri-, and quartenary-sialic acid moieties, respectively; while a and b denote structural isomers differing only in the position of one sialic acid.

Protein Binding Site

The protein receptor required for BoNT entry is not as well studied, though a recent report shows the synaptic vesicle protein SV2 is the protein receptor for BoNT/A [70], while other reports showed synaptotagmins I and II act as receptor to BoNT/B [71] and BoNT/G [66], and synaptotagmin I is bound by BoNT/A in the presence of specific gangliosides [67].

Despite these recent discoveries, there are still significant gaps in the knowledge required to develop effective countermeasures based on interfering with the BoNT/A binding site and prevention of toxin uptake into neuronal cells. Specifically,

1. A systematic binding study of multiple potential gangliosides and protein receptor has not been undertaken and resulted in limited structure-activity relationship data.
2. Specific binding constants of these gangliosides and protein receptor have not been determined (except for one specific compound, GT1b, against BoNT/A [64]).

3. The known crystal structure of the BoNT/A has not been reconciled with ganglioside or protein receptor binding in a cohesive *in silico* (computer-based) molecular model.

The mechanism of BoNT/A binding to neuron cells could be elucidated through:

1. Co-immunoprecipitation of BoNT HC with gangliosides and neuronal cell membrane extracts in conjunction with proteomics studies to identify the bound protein.
2. Use of μ-affinity MS to measure binding affinities of gangliosides and the identified protein receptor, thus obtain structure-activity-relationship data between BoNT and its receptors.
3. Determination of specific peptide sequence in BoNT/A HC responsible for binding.

With a greater understanding of BoNT/A HC binding mechanism, an *in silico* docking model can be applied to the development of inhibitors of BoNT/A binding to neurons.

Animal model for *in vivo* evaluation of drug candidates

In order to assess efficacy of drug candidates, the *in vivo* activity in an appropriate animal model is necessary. The mouse toxicity and neutralization bioassays that are currently used for detection and identification of BoNT could also be used as the drug efficacy animal model. An in-house mouse bioassay will be developed and used to:

1. determine the lethal dose to 50% of the population (LD_{50}) for BoNT/A.
2. determine efficacy of drug candidates from the drug discovery process (high throughput FRET, enzymatic activity based CE-LIF, binding based μ-affinity MS, and cell based assays).
3. determine the toxicology of each drug candidate.

In the in-house mouse bioassay [72], BoNT/A will be injected into mice intraperitoneally (i.p.). After injection, mice will be monitored for signs of stress such as ruffled fur, followed in sequence by laboured abdomincal breathing, limb weakness, total paralysis, and finally death by respiratory failure. The drug efficacy can be observed in the form of neutralization of the BoNT toxicity with i.p. injection of each drug candidate at various concentrations.

Conclusion

The BoNT R&D efforts presented in this technical memorandum showcase all the steps involved in comprehensive preclinical drug discovery studies. The drug discovery process begins with the identification of a medical need (botulinum poisoning), including a judgement on the adequacy of existing therapies (antitoxin administration alone is deemed inadequate). From this analysis, together with an appraisal of the current knowledge about the target disease (inhibitors against BoNT LC, and identification of cell surface receptors for BoNT), hypotheses will be formed on how to possibly improve current therapy – that is, what efficacy, safety or mechanistically novel improvements will advance the method of drug treatment for patients with the target disease? Our answer to that question will be in the form of a combination of antitoxin and drugs against cell binding and the proteolytic activity of BoNT. On the basis of these hypotheses, specific objectives can be set for the future work. Key steps in the drug discovery process include detecting relevant biological activity for a structurally novel compound *in vitro* (high throughput FRET, enzymatic activity based CE-LIF, binding based μ-affinity MS, and cell based assays), then finding a candidate with *in vivo* activity in an appropriate animal model (mouse LD_{50}), followed by maximizing this activity through the selection of analogous structures (*in silico* docking), and finally selecting a compound as the candidate for drug development. This drug candidate can then undergo toxicological testing in animals prior to clinical studies.

In spite of recent news coverage of botulinum poisoning from consumption of commercial organic carrot juice in the United States and Canada in September 2006, there remains little known about the mechanism of botulinum neurotoxin binding, or development of effective drug candidate, and delays in correct diagnosis of botulinum poisoning. Fast and proper diagnosis of botulinum poisoning (rapid detection based on protein fingerprinting), and new strategic therapies are needed in addition to the current protocol for treatment of botulinum poisoning: administration of antitoxin, adequate mechanical ventilation, and often meticulous and intensive care.

The overview presented in this article was directed toward BoNT. The model created for drug discovery and countermeasure development is applicable to other toxins such as tetanus, ricin, and anthrax.

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List of symbols/abbreviations/acronyms/initialisms

BoNTs	Botulinum neurotoxins
CBQCA	3-(4-carbozylbenzoyl)-2-quinoline carboxaldehyde
CCD	Charge-coupled device
CDC	Centers for Disease Control and Prevention
CE-LIF	Capillary electrophoresis with laser induced fluorescence
Da, kDa	Dalton, Kilodalton
DMPS	2, 3-dimercaptopropanesulfonate
DMSO	Dimethylsulfoxide
Dnp	2, 4-dinitrophenol
DRDC	Defence Research & Development Canada
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EIC	Extracted ion chromatogram
FRET	Fluorescence resonance energy transfer
GABA	γ-Aminobutyric acid
HC	Heavy chain
HPLC	High performance liquid chromatography
IC_{50}	Concentration of inhibitor that reduces enzyme velocity by half
IgG-HRP	Immunoglobulin G fused with horse radish peroxidase
K_d	Dissociation constant

K_I	Inhibition constant
K_M	Michaelis-Menten constant
LC	Light chain
LC-ESI-MS	Liquid chromatography electrospray ionization mass spectrometry
LD_{50}	Lethal dose causing death of 50% of population
m/z	Mass-to-charge
MSA	Mercaptosuccinic acid
MW	Molecular weight
o-Abz	Ortho-aminobenzoic acid
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PVDF	polyvinylidene difluoride
PWGSC	Public Works and Government Service Canada
R&D	Research and development
RP	Reversed phase
SDS-PAGE	Sodium dodecyl (lauryl) sulphate – polyacrylamide gel electrophoresis
SNAP-25	Synaptosomal-associated protein of 25 kDa
SNARE	Soluble NSF (N-ethylmaleimide-sensitive fusion protein)-attachment protein receptor
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TIF	Technology investment fund
UV	Ultraviolet light
WHO	The World Health Organization

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In September 2006, several cases of botulinum poisoning were reported in United States and Canada due to consumption of commercial organic carrot juice. This incident led to the hospitalization of several individuals who received intensive ventilator support. In spite of botulinum neurotoxin being the most poisonous material, little is known about its mechanism of binding, effective drugs are lacking, and correct diagnosis of botulinum poisoning is slow. Fast and accurate diagnosis of botulinum poisoning (through protein fingerprinting), and new rational drug designs are needed to supplement the current protocol for treatment of botulinum poisoning: administration of antitoxin, adequate mechanical ventilation, and meticulous and intensive care. This technical memorandum reviews past and present research & development efforts on botulinum neurotoxins and future pre-clinical drug discovery directions at DRDC Suffield. A comprehensive drug discovery process is described, including high throughput screening FRET assay, rapid and efficient CE-LIF enzymatic assay, equilibrium binding μ-affinity MS assay, cell-based assay, and *in vivo* mouse bioassay. Mechanistically-novel additions to current therapeutics could be in the form of a combination of antitoxin and drugs against cell binding and/or the proteolytic activity of botulinum neurotoxin.

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