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High Throughput Screening of Pharmacologically Active Compounds Against Botulinum Neurotoxin Serotype A Light Chain

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Abstract

Botulinum neurotoxin (BoNT) is the most toxic natural substance known and has long been considered a potential warfare agent. More recently, it has also been considered a growing bioterrorism threat. Unfortunately, there is no effective cure for poisoning, regardless of whether this is a result of deliberate exposure or accidental ingestion of naturally contaminated foodstuffs. Effective countermeasures against BoNT might be achieved byidentifying small molecule inhibitors against the toxin’s enzymatic active site. To this end, a high throughput screening (HTS) assay based on fluorescence resonance energy transfer (FRET) was established to interrogate small molecule compounds from two proprietary sets of chemical libraries against BoNT serotype A light chain (BoNT/A LC). The chemical libraries were LOPAC (the Library of Pharmaceutically Active Compounds) and the Prestwick Chemical Library (a collection of off-patent drugs). FRET assay buffer conditions were optimized for fast substrate turnover in short incubation time and low background noise. Chemical compound endogenous fluorescence and absorption at 321 nm were measured to address possible interferences. Potential inhibitors and activators of BoNT/A LC were compiled for further comprehensive studies in computational molecular modeling, dose response and enzyme kinetics. Using this HTS method, some 2400 compounds were screened in less than 4 months with minimal instrumentation requirement.

Résumé

La neurotoxine botulique (BoNT), la plus toxique des substances naturelles connues, est considérée depuis longtemps comme un agent de guerre potentiel. Plus récemment, elle est également considérée comme une menace bioterroriste grandissante. Il n’existe malheureusement aucun remède efficace contre ce type d’empoisonnement, que ce soit le résultat d’une exposition délibérée ou de l’ingestion accidentelle d’aliments naturellement contaminés. Il serait toutefois possible de concevoir des contre-mesures efficaces à la BoNT en identifiant de petites molécules ayant un effet inhibiteur contre le site d’activité enzymatique de la toxine. À cette fin, nous avons mis au point un test de criblage à haut débit basé sur le transfert d’énergie de fluorescence par résonance (FRET) pour mettre à l’essai des composés à petites molécules provenant de deux ensembles exclusifs de bibliothèques de composés chimiques contre la chaîne légère de la BoNT de sérotype A (BoNT/A LC). Nous avons procédé au criblage de deux bibliothèques de composés chimiques : la LOPAC (Library of pharmaceutically active compounds) et la bibliothèque de médicaments hors brevet Prestwick. Les conditions de tampon de l’épreuve FRET ont été optimisées pour obtenir un renouvellement rapide du substrat dans un court temps d’incubation et avec un faible bruit de fond. Nous avons mesuré la fluorescence endogène et l’absorption à 321 nm des composés chimiques afin d’éliminer les interférences possibles. Nous avons compilé une liste d’inhibiteurs et d’activateurs possibles de la BoNT/A LC en vue d’études plus approfondies de modélisation moléculaire computationnelle, de dose-réponse et de cinétique enzymatique. Cette méthode de criblage à haut débit a permis d’analyser quelque 2 400 composés en moins de quatre mois avec une exigence minimale en matière d’instrumentation.

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Executive summary

High Throughput Screening of Pharmacologically Active Compounds Against Botulinum Neurotoxin Serotype A Light Chain

Nora W.C. Chan; William E. Lee; Jessica Wong; Melissa Crichton; David C.W. Mah; DRDC Suffield TM 2008-252; Defence R&D Canada – Suffield; November 2008.

Background: Botulinum neurotoxin (BoNT) is the most toxic natural substance known. Ingestion of foodstuffs contaminated with BoNT results in the paralytic disease botulism; inhalational exposure is also deadly. BoNT has long been considered a potential biological warfare agent (agent X); more recently, it has also been considered a growing bioterrorism threat, due to increasing availability of large-scale protein expression. Unfortunately, there is currently no effective cure for BoNT poisoning, regardless of whether this is a result of deliberate exposure or accidental ingestion of naturally contaminated foodstuffs.

Results: To address this biodefence gap, some 2400 compounds already known to be pharmacologically active were screened for their activity towards a component of BoNT that attacks essential proteins in nerve cells (the "light chain"), using a high throughput screening assay developed at DRDC Suffield. The majority of the compounds produced no effect on the activity of the BoNT light chain. However, roughly 1% of the compounds screened (23 in total) produced significant inhibition; a comparable number app to enhance the activity of the light chain.

Significance: Prior to this work, there had been very few reports of the inhibition of the BoNT light chain by small, "drug-like" compounds, too few to serve as a basis for drug development. This work has substantially increased the number of such compounds and could serve as the first step in a long drug discovery process to identify a drug to counter the effects of botulism in humans.

Future plans: Further efforts will focus on detailed studies to determine concentration response, binding affinities, and inhibition mechanisms of each inhibitor. Computational molecular modeling of the interaction between inhibitors with the BoNT light chain could provide insights into the mechanisms of inhibition and thus lead to improved inhibitor designs.

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High Throughput Screening of Pharmacologically Active Compounds Against Botulinum Neurotoxin Serotype A Light Chain

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Contexte : La neurotoxine botulique (BoNT) est la plus toxique des substances naturelles connues. L'ingestion d'aliments contaminés par la BoNT provoque le botulisme, une maladie paralytique; l'exposition par inhalation est également mortelle. La BoNT est considérée depuis longtemps comme un agent de guerre biologique potentiel (agent X) et, depuis peu, comme une menace bioterroriste grandissante en raison de la disponibilité croissante de l'expression de ses protéines à grande échelle. À l'heure actuelle, il n'existe malheureusement aucun remède efficace contre l'empoisonnement par la BoNT, que ce soit le résultat d'une exposition délibérée ou de l'ingestion accidentelle d'aliments naturellement contaminés.

Résultats : Pour combler cette lacune sur le plan de la défense biologique, quelque 2 400 composés déjà connus pour être pharmacologiquement actifs ont été mis à l'essai pour leur activité à l'égard d'un composant de la BoNT qui attaque les protéines essentielles des cellules nerveuses (la « chaîne légère ») au moyen d'un test de criblage à haut débit mis au point par RDDC Suffield. La majorité des composés n'ont produit aucun effet sur l'activité de la chaîne légère de la BoNT. Toutefois, environ 1 % des composés criblés (23 au total) ont présenté une activité inhibitrice significative; un nombre comparable a semblé améliorer l'activité de la chaîne légère.

Importance : Avant les présents travaux, il y avait très peu de rapports d'études sur l'inhibition de la chaîne légère de la BoNT par de petits composés s'apparentant à un médicament, trop peu pour servir de fondement à la mise au point de médicaments. Ces travaux ont fait croître considérablement le nombre de composés de ce genre et pourraient constituer la première étape d'un long processus de recherche visant à trouver un médicament capable de contrer les effets du botulisme chez l'humain.

Perspectives : Des efforts supplémentaires seront centrés sur des études détaillées pour déterminer la relation concentration-effet, les affinités de liaison et les mécanismes d'inhibition de chaque inhibiteur. La modélisation moléculaire computationnelle de l'interaction entre les inhibiteurs et la chaîne légère de BoNT pourrait donner un aperçu des mécanismes d'inhibition et ainsi améliorer la conception de ces inhibiteurs.

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Introduction

Botulinum neurotoxin (BoNT), of which there are seven serotypes (designated A through G), is the most toxic substance known (intravenous LD50 is 1 ng/kg) [1]. Serotypes A, B, E, and F have been identified in numerous human poisoning episodes [2]. Botulinum neurotoxin consists of a light chain (LC, 50 kDa) and a heavy chain (HC, 100 kDa) covalently linked together by a single disulfide bond [3]. The heavy chain is responsible for irreversible binding to a cell surface receptor on nerve cells prior to entering through endocytosis. The light chain is a zinc-dependent endopeptidase [4–7]. 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 the neurotransmitter acetylcholine [8, 9]. BoNT blocks the autonomic postganglionic terminals from releasing acetylcholine, resulting in muscle paralysis. In the central nervous system, BoNT inhibits the release of a wide range of neurotransmitters due to its ability to cleave SNARE proteins.

In the past few years, BoNT R&D efforts at DRDC Suffield have focused on screening combinatorial peptide libraries as potential inhibitors, as the structural and conformational information may allow the design of better small molecule drugs against BoNT [10–14]. Research was done primarily with in vitro assays to study the inhibitory effects of combinatorial peptides (typically tetra- or tri-peptide molecules) on BoNT/A and BoNT/B. The advantages of small molecules over peptides as inhibitors are better bioavailability due to resistance from proteases in digestive and circulatory systems, and easier transport across cell membranes into the nerve terminal where BoNT LC exerts it toxic effects. Small molecule (non-peptidic) therapeutics have not been found to counter BoNT poisoning. There are limited reports of small-molecule inhibition of BoNT/A LC by captopril [10, 14], bisquinolines [15], and bis-imidazoles [16]. A serotype-selective inhibitor was also recently identified [17]. The small molecule libraries studied in this work consisted of pharmacologically active compounds and/or off-patent drugs are useful in the early stage high throughput drug screening process. Pharmacologically active compounds are categorized into different classes of receptor antagonists and enzyme inhibitors that will facilitate the study of structure-activity relationship between BoNT/A LC and its inhibitors. Off-patent drugs are particularly useful in accelerating the drug development process since bioavailability and human safety information are readily available from previous clinical trials.

A high throughput screening (HTS) assay, which is fast and easy, with relatively low sample consumption, is required for screening thousands of compounds in a short period of time. 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 long range intramolecular dipole-dipole coupling [18, 19]. This process allows for development of various homogenous enzymatic assays where fluorescence resulting in cleavage of a FRET substrate is monitored and directly correlated to the enzyme activity, one of which is the commercially available SNAPtide assay. Massively parallel FRET enzymatic assays (in 96-well plate) can be done because physical separation of the product from the substrate is not required and therefore is ideal for HTS assay of enzyme inhibitors.

SNAPtide is the synthetic peptide substrate specifically designed for a FRET assay to measure BoNT/A LC activity (figure 1). SNAPtide contains the sequence of the natural protein target,

SNAP-25, 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). Intact SNAPtide does not fluoresce due to the proximity of the quencher to the fluorescent dye. As soon as the peptide is cleaved at the **QR** site, the truncated peptide produces a fluorescent signal. This assay system provides a high throughput screening method for testing two proprietary sets of compound libraries. First is the library of pharmacologically active compounds (LOPAC) , which contains 1280 compounds in collections of sixteen 96-well plates of 80 compounds per plate. Second is the Prestwick off-licensed drug library of fourteen 96-well plates of 80 compounds per plate. This FRET assay will be used as a preliminary step, where each set can be screened for inhibition to identify sample(s) containing potential inhibitors. In the future, a “drill-down” process will allow for deconvolution of the library sets, and eventually individual testing of compounds from the active set to determine IC_{50} (concentration of inhibitor that reduces enzyme velocity by half), binding affinity and enzyme kinetics.

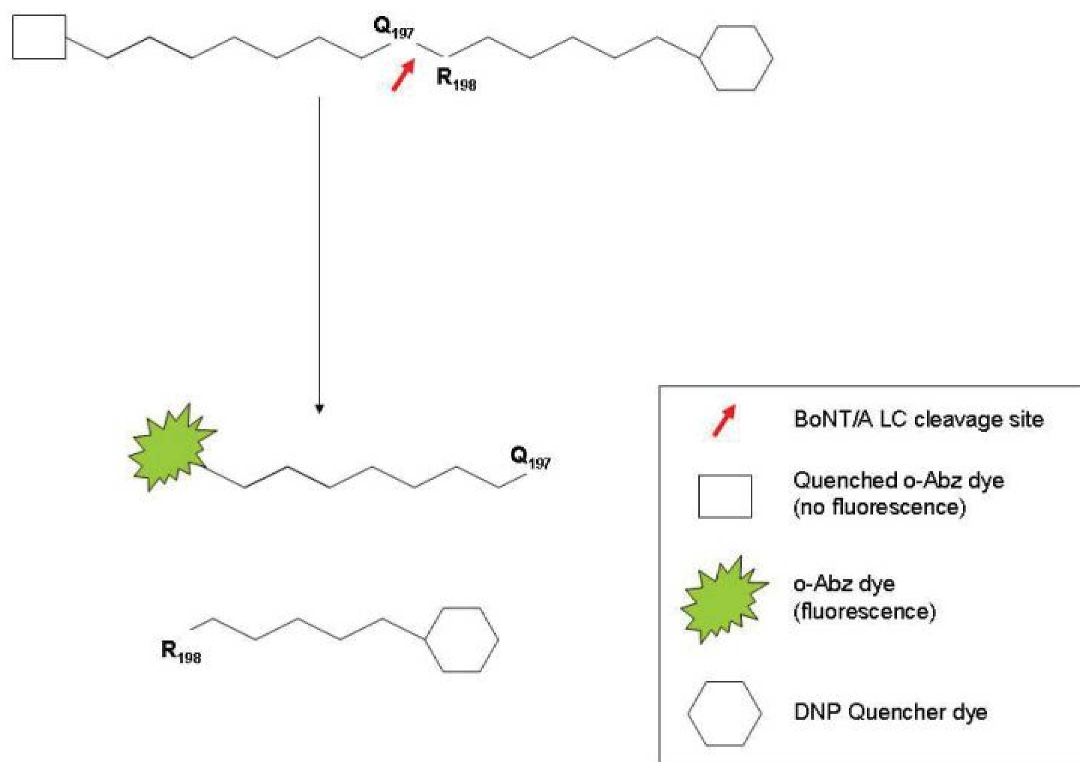

Figure 1: Schematic of a SNAPtide assay.
SNAPtide substrate containing a quenched fluorescent dye, o-aminobenzoic acid (o-Abz), and quencher, 2,4-dinitrophenol (DNP) has the cleavage site for BoNT/A LC. After incubation and the fluorescent dye is released from the quencher, o-Abz fluoresces at 418 nm with an excitation wavelength at 321 nm.

Materials and Methods

All reagents are from Sigma-Aldrich Co. (Oakville, ON) unless indicated otherwise. The FRET substrate, o-Abz/DNP-SNAPtide (5 µM), was mixed with 5 nM BoNT/A LC in assay buffer (50 mM HEPES buffer, 0.3 mM $ZnSO_4$, 1 mg/mL BSA, and 0.5 mM dithiothreitol (DTT), pH 8.0) to a final assay volume of 100 µL. Assay conditions were optimized for sample volume per well, assay buffer components and incubation time. The SNAPtide substrate and recombinant BoNT/A LC are commercially available from List Biological Laboratories, Inc. through Cedarlane Ltd. (Burlington, ON). The reaction mixture was incubated at 37 °C for 60 min, and then stopped with the addition of 500 µL assay stop solution (10 mM Tris, pH 8.0, 100 mM EDTA). Each sample was divided and 275 µL aliquots transferred into two separate microplate wells. Fluorescence was measured on a fluorescence spectrophotometer with a 96-well microplate accessory (Cary Eclipse, Varian Canada Inc., Mississauga, ON) with excitation wavelength at 321 nm, and emission wavelength at 418 nm. Costar black round bottom 96-well plates (Corning, NY) were used for all assays. An unquenched SNAPtide calibrant containing the same fluorescently-labelled peptide sequence without the quencher (DNP) was used to generate a standard curve to calculate the percentage substrate turnover in each positive control. A negative control (which did not contain enzyme) was used to determine the background noise level of the FRET assay.

Standard calibration curve was obtained using a unquenched SNAPtide standard (SNAPtide without DNP). A range of standard concentrations, 0 – 1000 µM, were used. Percent substrate turnover was calculated from the fluorescent signal from each assay sample and the standard curve.

The LOPAC library was supplied as individual compounds dissolved in DMSO at a concentration of 10 mM. Daughter plates were prepared by diluting all compounds in water to a concentration of 1 mM in 10% DMSO. Similarly, the Prestwick off-patent drug library was supplied as individual compounds dissolved in DMSO at a concentration of 2 mg/mL. Daughter plates were prepare by 1/5 dilution in water to a concentration of 0.4 mg/mL. An aliquot of 10 µL per compound was added to the reaction mixture for each test compound. Each compound was tested in duplicates. Appropriate amounts of DMSO (10% or 20%) were added to positive and negative controls to account for the effects of DMSO on BoNT/A LC activity. The final amount of DMSO in each assay did not exceed 2%, which was tolerated by BoNT/A LC.

Selected compounds from both libraries were diluted in a solution of assay buffer/assay stop solution (1:5) for background fluorescence measurements and absorbance measurements at 321 nm.

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Results and Discussion

Assay Optimization

The BoNT/A LC SNAPtide assay was optimized for high throughput screening of pharmacologically active compounds against the light chain subunit of BoNT for volume and buffer as follows.

As shown in Table 1, the sample volume in each 96-well was important. When the volume was below 250 µL per well, the background fluorescence signal was very high due to light scattering. By employing a standard sample volume of 275 µL per well for all assays, the systematic background fluorescence was minimized.

Table 1: Relative fluorescence unit (RFU) measured of different assay buffer volumes per well in Costar 96-well plate

Sample volume (µL) per well	Background fluorescence signal (RFU)
200	171.6 ± 46.4
250	22.31 ± 0.728
275	14.35 ± 1.14

The FRET assay buffer condition was also optimized for maximum substrate turnover at a fixed incubation time. The assay buffer, 50 mM HEPES buffer, pH 8.0 with 0.3 mM $ZnSO_4$, and 0.5 mM DTT, containing either 1 mg/mL BSA or 0.1% Tween-20 was evaluated. The reaction mixture containing 5 µM o-Abz/DNP-SNAPtide substrate and 5 nM BoNT/A LC was incubated in each buffer. The buffer containing BSA resulted in higher substrate turnover rate (see Table 2) and was used in subsequent assays.

Table 2: Substrate turnover in BoNT/A LC FRET assay in buffer containing 1 mg/mL BSA compared to that with 0.1% Tween-20

Time (min)	Percentage of turnover of substrate	
	Buffer with BSA	Buffer with Tween-20
0	0	0
5	4.81	3.18
15	12.6	5.15
30	20.8	6.67

A linear fluorescence signal dependent on the concentration of unquenched standard was obtained (Figure 2). The calibration curve was reproduced daily within 10% error (data not shown). The linear equation resulting from the calibration curve was used to calculate the amount of product formed in each assay. Thus percentage substrate turnover can be calculated as a fraction of the amount of product formed from the initial amount of substrate in each assay. Typically, the assay is designed to turnover about 50 - 70% of the substrate to product (1 hour incubation at 37 °C). This level of substrate turnover provided a good base for assessing efficacies of pharmacologically active compounds in the inhibition studies (an inhibitor reduces percentage turnover).

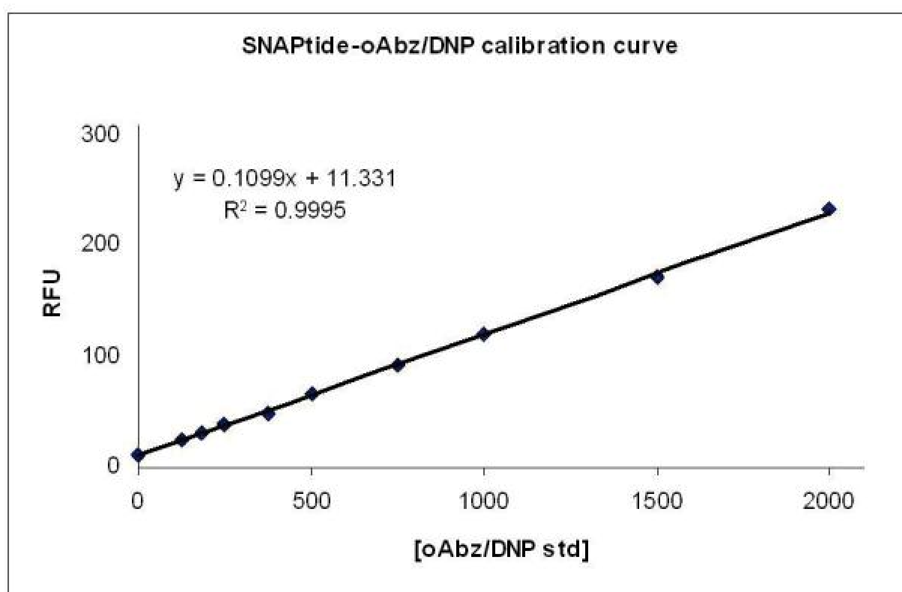

Figure 2: A typical calibration curve of the unquenched SNAPtide standard, showing linear correlation between the concentration of unquenched SNAPtide substrate and the fluorescent signal.

In each assay, a positive control (0% inhibition) containing appropriate amount of DMSO was used to account for variations in BoNT/A LC activity. Background fluorescence at 418 nm and absorbance at 321 nm of each small molecule compound were measured in a solution of assay buffer:assay stop solution (1:5). The measurement of background fluorescence (λ_{ex} 321, λ_{em} 418) will account for fluorescence signals endogenous to the small molecule compound that may interfere with o-Abz/DNP SNAPtide product signals and result in false activity enhancement. The measurement of absorbance at 321 nm accounts for a “filter effect” whereby the compound absorbs energy at 321 nm and reduces excitation of SNAPtide product resulting in false inhibition.

Inhibitors

After screening 2400 compounds, a list of potential BoNT/A LC inhibitors was generated (see table 3). The percentage inhibition is the calculated decrease in amount of product formed in the presence of 100 μM inhibitor in the FRET assay. The list of inhibitors was selected based on three criteria: more than 50% inhibition, low background fluorescence, and low absorbance at 321 nm.

Further studies, such as concentration-dependent inhibition, IC_{50}, enzyme kinetics for mode of inhibition, binding affinities, does response curves in cell and animal studies, and computational molecular modeling, will be required to investigate these potential inhibitors.

Table 3: List of potential inhibitors of BoNT/A LC.

Compound name	% Inhibition ± standard deviation
Rotenone	65 ± 3.2
SB 224289 hydrochloride	66 ± 5.6
Alexidine dihydrochloride	66 ± 2.0
Phenazopyridine hydrochloride	67 ± 1.7
Levocabastine hydrochloride	68 ± 1.3
Fiduxosin hydrochloride	69 ± 3.2
Diacylglycerol kinase inhibitor II	69 ± 6.2
Ruthenium red	70 ± 3.6
Propidium iodide	70 ± 2.5
Retinoic acid	71 ± 10
Mifepristone	74 ± 1.9
Methoxy-6-harmalan	76 ± 3.1
Verteporfin	76 ± 3.8
Calmidazolium chloride	77 ± 5.8
Tyrphostin AG 112	77 ± 5.0
Benzethonium chloride	77 ± 2.2
Piperine	81 ± 7.3
Atovaquone	81 ± 4.0
Tolmetin sodium salt dehydrate	82 ± 6.4
Apigenin	83 ± 13
Retinoic acid p-hydroxyanilide	86 ± 4.6
Cephalexin hydrate	99 ± 2.2
Raloxifene hydrochloride	99 ± 1.7

Activators

A list of potential activators of BoNT/A LC was generated, table 4, based on an increase in fluorescence signals in the FRET assay. The value of percentage enhancement was calculated from increase in FRET fluorescence signal with endogenous background fluorescence subtracted.

Activation is an interesting phenomenon. It is harder to conceptualize than inhibition. Activation of BoNT/A LC was observed in previous work [13, 14] where combinatorial peptide libraries were tested against reduced BoNT/A and HPLC separation of substrate and products. So, there is a low likelihood that all activation observed here is artificial.

Activators may not be useful as medical countermeasures against BoNT/A, though the structural information as to how these potential activators affects BoNT/A LC could be exploited for use in a rational drug design. The computational molecular modeling information compared between BoNT/A LC inhibitors and activators could provide insights into the enzymatic mechanism of BoNT/A LC and thus lead to improved inhibitor design.

Table 4: List of potential activators of BoNT/A LC.

Compound name	% enhancement ± standard deviation
Scoulerine	20 ± 2.8
Trigonelline	20 ± 3.0
Vancomycin hydrochloride	21 ± 2.3
Flucloxacillin sodium	21 ± 4.7
Prednicarbate	21 ± 5.9
Metergoline	21 ± 6.1
Betonicine	22 ± 0.46
Doxorubicin hydrochloride	23 ± 7.2
Nitrendipine	24 ± 3.5
Solasodine	24 ± 5.9
Xamoterol hemifumarate	25 ± 5.5
Hydrocotarnine hydrobromide	26 ± 3.0
Hydrocortisone base	27 ± 10
Monocrotaline	27 ± 4.3
Niacin	27 ± 4.7
Berberine chloride	27 ± 6.8
Gabexate mesilate	27 ± 7.5
Meptazinol hydrochloride	28 ± 2.9
Yohimbine hydrochloride	28 ± 3.8
Idazoxan hydrochloride	28 ± 4.4
Pilocarpine nitrate	28 ± 5.2
Equilin	31 ± 4.0
Altretamine	31 ± 6.0
3-Isobutyl-1-methylxanthine	33 ± 14
alpha-Santonin	33 ± 2.1
Decamethonium bromide	33 ± 3.2
Chloropyramine hydrochloride	33 ± 7.5
Denatonium benzoate	35 ± 5.1
Austricine hydrate	35 ± 8.3
Myosmine	36 ± 7.1
Karakoline	36 ± 9.3
Alfaxalone	37 ± 7.5
Alfadolone acetate	39 ± 11
Demecarium bromide	41 ± 7.3
Glafenine hydrochloride	42 ± 21
Reserpinic acid hydrochloride	45 ± 8.5
SB-366791	56 ± 12
Phloretin	59 ± 17
3-Methyl-6-(3-[trifluoromethyl]phenyl)-1,2,4-triazolo[4,3-b]pyridazine	79 ± 16
Cilostamide	80 ± 12
Eserine ulphate, physostigmine sulphate	85 ± 10
Ofloxacin	85 ± 15
Methyl beta-carboline-3-carboxylate	92 ± 11
Pseudopelletierine hydrochloride	99 ± 17
Graveoline	102 ± 17
1-Methyl-N-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1H-indazole-3-carboxamide maleate	112 ± 16
Ethamsylate	117 ± 11

Conclusion

Botulinum neurotoxin (BoNT) is the most toxic natural, known substance that can cause poisoning and there is no effective cure. There is an urgent need to design medical countermeasures against BoNT. Developments of small molecule drugs are desirable because they provide lower cost, higher quality, greater availability and longer shelf-life stability over immunotherapy. Two sets of chemical libraries were tested against BoNT/A LC. The LOPAC library consists of pharmacologically active compounds that are categorized into different classes of receptor antagoists and enzyme inhibitors could facilitate the study of structural-activity relationship between BoNT/A LC and its inhibitors. The Prestwick library consists of off-patent drugs could accelerate the drug development process in bypassing bioavailability and human safety studies since these information are readily available from previous clinical trials.

A high throughput screening assay was developed for testing inhibitors of BoNT/A light chain (BoNT/A LC). This FRET assay requires minimal sample volume (100 μL), reagent consumption, and sample processing in a massively parallel manner (96-well fluorescence plate reader). In less than 4 months, 2400 compounds were screened in the BoNT/A LC FRET assay. This FRET assay has proven to be an effective and dependable first step in a drug discovery process.

The BoNT/A LC assay is susceptible to up and down modulations as observed here and in previous studies. A list of potential inhibitors of BoNT/A LC was generated based on a threshold of 60% inhibition at 100 μM inhibitor concentration in the assay. A list of potential activators was generated also. Each list has approximately 1% of total screened compounds. For a useful prospective drug discovery platform, a typical hit rate ranges from 0.1% to 10% depending on stringency of the assay [20-23].

Further studies, such as concentration-dependent inhibition, IC_{50} values, enzyme kinetics for mode of inhibition, binding affinities, dose response curves in cell and animal studies, and computational molecular modeling, will be required to investigate these potential inhibitors. Molecular modeling into the structural interaction between activators and BoNT/A LC and that of inhibitor and BoNT/A LC could be exploited for rational drug design.

Microbiological origin and mode of action of botulism, tetanus, anthrax, and ricin are different but they share many similarities in protein size and conformations, and in topology of mechanism. The R&D process learned from this project would give DRDC a new capacity for developing countermeasures against all other toxins such as anthrax, tetanus, and ricin.

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References

[1] Franz, D.R. (1997), Defense against toxin weapons. In Sidell, F.R.; Takafuji, E.T.; and Franz, D.R. (eds), *Medical Aspects of Chemical and Biological Warfare*, Borden Institute, Washington, DC, pp. 603–619; see p. 607.

[2] Centers for Disease Control and Prevention, National Center for Infectious Diseases, Division of Bacterial and Mycotic Diseases (1998). *Botulism in the United States, 1899–1996. Handbook for epidemiologists, clinicians, and laboratory workers*. U.S. Department of Health and Human Services.

[3] Hatheway, C.L. (1990), Toxigenic clostridia, *Clinical Microbiology Reviews*, 3, 66–98.

[4] Binz, T.; Kurazono, H.; Wille, M.; Frevert, J.; Wernars, K.; and Niemann, H. (1990), The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins, *Journal of Biological Chemistry*, 265, 9153–9158.

[5] Hauser, D.; Eklund, M.W.; Kurazono, H.; Binz, T.; Niemann, H.; Gill, D.M.; Boquet, P.; and Popoff, M.R. (1990), Nucleotide sequence of *Clostridium botulinum* C1 neurotoxin. *Nucleic Acids Research*, 18, 4924.

[6] Binz, T.; Kurazono, H.; Popoff, M.R.; Eklund, M.W.; Sakaguchi, G.; Kozaki, S.; Krieglstein, K.; Henschen, A.; Gill, D.M.; and Niemann, H. (1990), Nucleotide sequence of the gene encoding *Clostridium botulinum* neurotoxin type D, *Nucleic Acids Research*, 18, 5556.

[7] Schiavo, G.; Matteoli, M.; and Montecucco, C. (2000), Neurotoxins affecting neuroexocytosis, *Physiological Reviews*, 80, 717–766.

[8] Blais, J.; Chapman, E.R.; Link, E.; Binz, T.; Yamasaki, S.; De Camilli, P.; Südhof, T.C.; Niemann, H.; and Jahn, R. (1993), Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25, *Nature*, 365, 160–163.

[9] Deloye, F.; Schiavo, G.; Doussau, F.; Rossetto, O.; Montecucco, M.; and Poulain, B. (1996), Molecular mechanisms of tetanus and botulinum neurotoxins, *Médecine/Sciences*, 12, 175–182.

[10] Hayden, J.; Pires, J.; Roy, S.; Hamilton, M.; and Moore, G.J. (2003), Discovery and design of novel inhibitors of botulinus neurotoxin A: Targeted ‘hinge’ peptide libraries, *Journal of Applied Toxicology*, 23, 1–7.

[11] Moore, G.J. (1997), ‘Hinge’ peptide libraries in basic research and drug development: Application to breast cancer and multiple sclerosis, *Drug Development Research*, 42, 157–163.

[12] Moore, G.J.; Smith, J.R.; Baylis, B.W.; and Matsoukas, J. (1995), Design and pharmacology of peptide mimetics, *Advances in Pharmacology*, 33, 91–141.

[13] Lee, W.E.; Chan, N.W.C.; Marenco, A.J.; Hamilton, M.G.; Moore, G.J.; Moore, D.; Hayden, L.J.; Dickinson-Laing, T.; Gregory, M.; and Mah, D.C.W. (2006), Toxin inhibition: Deconvolution strategies and bioassay screening of combinatorial peptide libraries, (DRDC Suffield TR 2006-192) Defence R&D Canada – Suffield.

[14] Moore, G.J.; Moore, D.M.; Roy, S.S.; Hayden, L.J.; Hamilton, M.G.; Chan, N.W.C.; and Lee, W.E. (2006), Hinge peptide combinatorial libraries for inhibitors of botulinum neurotoxins and saxitoxin: Deconvolution strategy, *Molecular Diversity*, 10, 9–16.

[15] Burnett, J.C.; Schmidt, J.J.; Stafford, R.G.; Panchal, R.G.; Nguyen, T.L.; Hermone, A.R.; Vennerstrom, J.L.; McGrath, C.F.; Lane, D.J.; Sausville, E.A.; Zaharevitz, D.W.; Gussio, R.; and Bavari, S. (2003), Novel small molecule inhibitors of botulinum neurotoxin A metalloprotease activity, *Biochemical and Biophysical Research Communications*, 310, 85–94.

[16] Merino, I.; Thompson, J.D.; Millard, C.B.; Schmidt, J.J.; and Pang, Y.-P. (2006), Bis-imidazoles as molecular probes for peripheral sites of the zinc endopeptidase of botulinum neurotoxin serotype A, *Bioorganic and Medicinal Chemistry*, 14, 3583–3591.

[17] Park, J.G.; Sill, P.C.; Makiyi, E.F.; Garcia-Sosa, A.T.; Millard, C.B.; Schmidt, J.J.; and Pang, Y.-P. (2006), Serotype-selective, small-molecule inhibitors of the zinc endopeptidase of botulinum neurotoxin serotype A, *Bioorganic and Medicinal Chemistry*, 14, 395–408.

[18] Förster, T. (1946),[Energy transport and fluorescence], *Naturwissenschaften*, 6, 166–175.

[19] Lakowicz, J.R. (1983). *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, chapter 10.

[20] Katz, D.; Ito, E.;, Lau, K.S.; Mocanu, J.D.; Bastianutto, C.; Schimmer, A.D.; and Liu, F.F. (2008), Increased efficiency for performing colony formation assays in 96-well plates: Novel applications to combination therapies and high-throughput screening, *Biotechniques*, 44(2), 9–14.

[21] Valley, M.P.; Zhou, W.; Hawkins, E.M.; Shultz, J.; Cali, J.J.; Worzella, T.;Bernad, L.; Good, T.; Good, D.; Riss, T.L., Klaubert, D.H.;and Wood, K.V. (2006), A bioluminescent assay for monoamine oxidase activity, *Analytical Biochemistry*, 359, 238–246.

[22] Polgár, T.; Baki, A.; Szendrei, G.I.; and Keseru, G.M. (2005), Comparative virtual and experimental high-throughput screening for glycogen synthase kinase-3β inhibitors, *Journal of Medicinal Chemistry*, 48, 7946–7959.

[23] Slack, M.; Kirchhoff, C.; Moller, C.; Winkler, D.; and Netzer, R. (2006), Identification of novel Kv1.3 blockers using a fluorescent cell-based ion channel assay, *Journal of Biomolecular Screening*, 11(1), 57–64.

List of symbols/abbreviations/acronyms/initialisms

o-Abz	o-Aminobenzoic acid
BoNTs	Botulinum neurotoxins
BSA	Bovine serum albumin
CB	Chemical biological
DMSO	Dimethylsulfoxide
DNP	2,4-Dinitrophenol
DRDC	Defence Research & Development Canada
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FRET	Fluorescence resonance energy transfer
HC	Heavy chain
HTS	High throughput screening
IC_{50}	Inhibitor concentration that reduces enzyme velocity by 50%
i.d.	Inner diameter
kDa	Kilodalton
LC	Light chain
LD_{50}	Lethal dose causing death of 50%
LOPAC	Library of pharmacologically active compounds
Q-R	Glutamine-arginine (1-letter amino acid designations)
RFU	Relative fluorescence unit
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors
$ZnSO_4$	Zinc sulfate

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Botulinum neurotoxin (BoNT) is the most toxic natural substance known and has long been considered a potential warfare agent. More recently, it has also been considered a growing bioterrorism threat. Unfortunately, there is no effective cure for poisoning, regardless of whether this is a result of deliberate exposure or accidental ingestion of naturally contaminated foodstuffs. Effective countermeasures against BoNT might be achieved byidentifying small molecule inhibitors against the toxin's enzymatic active site. To this end, a high throughput screening (HTS) assay based on fluorescence resonance energy transfer (FRET) was established to interrogate small molecule compounds from two proprietary sets of chemical libraries against BoNT serotype A light chain (BoNT/A LC). The chemical libraries were LOPAC (the Library of Pharmaceutically Active Compounds) and the Prestwick Chemical Library (a collection of off-patent drugs). FRET assay buffer conditions were optimized for fast substrate turnover in short incubation time and low background noise. Chemical compound endogenous fluorescence and absorption at 321 nm were measured to address possible interferences. Potential inhibitors and activators of BoNT/A LC were compiled for further comprehensive studies in computational molecular modeling, dose response and enzyme kinetics. Using this HTS method, some 2400 compounds were screened in less than 4 months with minimal instrumentation requirement.

La neurotoxine botulique (BoNT), la plus toxique des substances naturelles connues, est considérée depuis longtemps comme un agent de guerre potentiel. Plus récemment, elle est également considérée comme une menace bioterroriste grandissante. Il n'existe malheureusement aucun remède efficace contre ce type d'empoisonnement, que ce soit le résultat d'une exposition délibérée ou de l'ingestion accidentelle d'aliments naturellement contaminés. Il serait toutefois possible de concevoir des contre-mesures efficaces à la BoNT en identifiant de petites molécules ayant un effet inhibiteur contre le site d'activité enzymatique de la toxine. À cette fin, nous avons mis au point un test de criblage à haut débit basé sur le transfert d'énergie de fluorescence par résonance (FRET) pour mettre à l'essai des composés à petites molécules provenant de deux ensembles exclusifs de bibliothèques de composés chimiques contre la chaîne légère de la BoNT de sérotype A (BoNT/A LC). Nous avons procédé au criblage de deux bibliothèques de composés chimiques : la LOPAC (Library of pharmaceutically active compounds) et la bibliothèque de médicaments hors brevet Prestwick. Les conditions de tampon de l'épreuve FRET ont été optimisées pour obtenir un renouvellement rapide du substrat dans un court temps d'incubation et avec un faible bruit de fond. Nous avons mesuré la fluorescence endogène et l'absorption à 321 nm des composés chimiques afin d'éliminer les interférences possibles. Nous avons compilé une liste d'inhibiteurs et d'activateurs possibles de la BoNT/A LC en vue d'études plus approfondies de modélisation moléculaire computationnelle, de dose-réponse et de cinétique enzymatique. Cette méthode de criblage à haut débit a permis d'analyser quelque 2 400 composés en moins de quatre mois avec une exigence minimale en matière d'instrumentation.

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botulinum neurotoxin; medical countermeasures; high throughput screening; drug discovery

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