

***Recent Developments with Metalloprotease Inhibitor  
Class of Drug Candidates for Botulinum  
Neurotoxins***

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**Recent Developments with metalloprotease inhibitor class of drug candidates for  
Botulinum Neurotoxins**

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## **ABSTRACT**

Botulinum Neurotoxins are the most poisonous of all toxins with lethal dose in nanogram quantities. They are also potential biological warfare and bioterrorism agents due to their high toxicity and ease of preparation. On the other hand BoNTs are also being increasingly used for therapeutic and cosmetic purposes, and with that the chances of accidental overdose are increasing. And despite the potential damage they could cause to human health, there are no post-intoxication drugs available so far. But progress is being made in this direction. The crystal structures in native form and bound with substrate peptides have been determined, and these are enabling structure-based drug discovery possible. High throughput assays have also been designed to speed up the screening progress. Substrate-based and small molecule inhibitors have been identified. But turning high affinity inhibitors into clinically viable drug candidates has remained a challenge. We discuss here the latest developments and the future challenges in drug discovery for Botulinum neurotoxins.

**KEYWORDS: Bioterrorism, Clostridium botulinum, Neurotoxin, Drug discovery, Protease inhibitor.**

## 1. INTRODUCTION

Botulinum neurotoxins (BoNTs) are produced by anaerobic bacteria of the genus *Clostridium* (*C. botulinum*, *C. baratii* and some strains of *C. butyricum*) and are considered the most potent toxins known to humans so far. While the lethal dose of BoNTs for humans is not known, it has been estimated from the primate studies. And, by extrapolating the primate data, for a 70 kg human being, the lethal amounts of crystalline type A toxin would be 0.09-0.15 µg intravenously or intramuscularly, 0.70-0.90 µg by inhalation, and 70 µg orally [1]. BoNTs act by inhibiting the release of neurotransmitter, acetylcholine by peripheral nerve terminal which causes the flaccid paralysis and autonomic dysfunctions typical of botulism [2]. Because of their extreme toxicity they are considered a potential weapon of biological warfare and bioterrorism[1]. Ironically, they are also useful as therapeutic agents against human diseases involving hyperactive nerve terminals [3] and now they are increasingly being used for cosmetic purposes and sometimes leading to accidental overdose ending up in hospitalization and a lengthy recovery [4]. Currently, there is no pharmacological treatment for BoNT intoxication; affected patients are provided artificial ventilation.

There are six phylogenetically distinct clostridial groups that produce seven different serotypes (BoNT/A-G) and these serotypes are further divided into numerous subtypes on the basis of their amino acid sequences [5]. Among the seven serotypes of this neurotoxin, BoNT/A, B, E and possibly F cause toxicity in humans, BoNT/C in birds and BoNT/D in animals. Recently a new isolate has been designated as a novel serotype H but thorough experimental validation remains to be done to establish whether it is just a new subtype of the existing serotypes or indeed a new serotype [6-8].

## 2. DOMAIN ORGANIZATION OF BoNTs

A BoNT molecule is initially synthesized as a single polypeptide chain of ~150 kDa that gets proteolytically cleaved into two – a light chain (LC) of 50 kDa and a heavy chain (HC) of 100 kDa. The mature toxin consists of a light and a heavy chain, held together by a disulfide bond and a ‘belt’ from HC that wraps around LC providing most of the non-covalent interactions. The light chain (LC) has zinc dependent protease activity and it cleaves the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins and thus disrupts the acetylcholine release (Fig. 1). The HC consists of a C-terminal (HC<sub>C</sub>) domain responsible for presynaptic binding and endocytosis and consists of two subdomains - the N-terminal half, HC<sub>CN</sub> belonging to a Concanavalin-A like lectin superfamily and the C-terminal half, HC<sub>CC</sub> the receptor binding subdomain that recognizes the surface receptors on the pre-synaptic neuron ending. The N-terminal (HC<sub>N</sub>) translocation domain of the HC helps in translocation of the catalytic domain (LC) from endocytic vesicle into the neuronal cytosol [9],[10]. Each serotype of BoNT has specific protein target(s) in the SNARE complex and a specific peptide bond that it recognizes for cleavage. BoNT/A and E cleave SNAP-25 (synaptosomal-associated protein of 25 kDa); BoNT/B, D, F and G cleave VAMP (vesicle-associated membrane protein, also known as synaptobrevin); and BoNT/C cleaves syntaxin as well as SNAP-25.

## 3. MECHANISM OF ACTION

The most common form of botulism is food-borne botulism that occurs after the ingestion of BoNT-contaminated food e.g. canned food that contains pre-formed toxin. The toxin survives the proteolytic environment of the gastrointestinal tract by forming a tight complex with

NTNHA (non-toxic non-hemagglutinin) protein and is eventually absorbed in the intestines [11]. Infant botulism is caused by the consumption of food contaminated with clostridial spores that eventually germinate in the intestines and release the toxin [12]. The lack of a robust gut microbiota in infants compared to adults facilitates this process. The toxin eventually binds to the apical surface of the epithelial cells and is carried to the basal surface of cells lining the gut and released into the general circulation [13]. In the case of iatrogenic botulism the toxin reaches the blood circulation directly as a result of therapeutic or cosmetic injection and is typically a case of over-dose [4]. After getting into the blood circulation predominantly through the above-mentioned mechanisms BoNTs reach the peripheral cholinergic nerve terminals and the process of internalization starts. The binding of BoNTs is highly specific for peripheral nerve terminals and is based on dual receptor binding involving polysialogangliosides and a protein receptor (Synaptotagmin (Syt) or Synaptic vesicle protein 2 (SV2)) [14-17]. After getting attached, the BoNT is then endocytosed into synaptic vesicles. The vesicular ATPase proton pump causes the lowering of the vesicular pH leading to protonation of BoNT. The positively charged BoNT then interacts with the negatively charged vesicular membrane and the LC is released into the cytosol. In the final step, the LC owing to its protease activity binds and cleaves the proteins of the SNARE complex thus halting the neurotransmitter release causing flaccid paralysis. The onset of symptoms and the severity of the disease may depend upon the route of exposure, the exposure dose and the serotype but usually occurs within 72 hours [1]. A comprehensive review of the life cycle of BoNT producing bacteria and the mechanism of action of BoNT toxicity has appeared recently [18].

#### **4. ANTIBODIES AND VACCINES FOR BoNTs**

The first response upon the diagnosis of botulinum intoxication is the administration of botulinum antitoxin and artificial ventilation. The antitoxin neutralizes the BoNT flowing in the blood stream but it can't reach the fraction that has already entered into the neuronal cells. Also, the antitoxin is effective if given within 24 hours of appearance of the disease symptoms [19]. Approved by the US FDA in 2003, BabyBIG<sup>®</sup> is derived from the plasma pooled from human adults vaccinated with the pentavalent botulinum toxoid vaccine against serotypes A to E. It has been shown to significantly reduce the period of hospitalization and decrease the treatment costs [20]. A heptavalent botulinum antitoxin (H-BAT, Cangene Corp.) composed of <2% intact IgG and 90% Fab and F(ab')<sub>2</sub> immunoglobulin fragments became available in 2012 through a CDC-sponsored FDA Investigational New Drug (IND) protocol for the treatment of naturally acquired non-infant botulism [21]. H-BAT was subsequently found effective in treating a case of foodborne botulism without developing any hypersensitivity reactions or serum sickness. The patient was discharged to a long-term acute care facility on hospital day 22 [22]. A recombinant bivalent Hc vaccine against serotypes A1 and B1 (rBV A/B) produced in *P. pastoris* has shown very encouraging initial results and has undergone phase II clinical trials, a randomized, double-blind study to evaluate the safety and immunogenicity [23, 24]. But, as of September 19, 2014, the recruitment of volunteers for phase III clinical trials has been suspended as mentioned on the [www.clinicaltrials.gov](http://www.clinicaltrials.gov) website.

#### **5. TARGETING PROTEASE ACTIVITY OF BoNT FOR DRUG DISCOVERY**

Since symptoms of botulism appear only after BoNT light chain has started its protease activity in neuronal cytosol leading to blockage of neurotransmitter release, the antibodies and the vaccines are ineffective on this fraction of the toxin. Also, given the ever-increasing use of

BoNTs for various therapeutic and cosmetic purposes, *en masse* vaccination against this toxin is not advisable. For these reasons, the BoNT catalytic domain makes an ideal post-intoxication drug target. The atomic structure of BoNT catalytic domain has been determined for all serotypes from A to G enabling structure-based drug discovery. The catalytic domain of BoNT is a zinc dependent protease similar to thermolysin. The Zn(II) cofactor binds at the active site to a conserved HExxH + E motif found in all serotypes, coordinated by the side chains of two histidines and a glutamate. A fourth coordination is provided by a conserved water molecule that acts as a nucleophile. The glutamate acts as the general base in the catalytic mechanism of the enzyme. While the core structure of BoNT catalytic domain is consistent across serotypes, four flexible loops designated as the 50/60 loop, 170 loop, 250 loop and 370 lining the active-site are flexible, found in various conformations and participate in substrate binding. The BoNT catalytic domain shows 33 - 37% amino acid sequence identity among various serotypes and each serotype recognizes a unique peptide bond for cleavage. Among the three serotypes - A, B and E that infect humans, BoNT/A is the most lethal and frequently occurring serotype. Therefore, most of the drug discovery effort has been targeted against this serotype.

### 5.1 Development of assays for BoNT protease activity

Identification of inhibitors for any given target often involves high throughput screening of large libraries of compounds. In the case of BoNTs, several assays have been designed to screen compounds against its protease activity in an efficient manner. Schmidt and Bostian designed the early assays identifying a 17-mer peptide that could be used as a substrate instead of the full length SNAP-25 and addition of bovine serum albumin in the assay for optimum activity [25, 26]. Later, Schmidt and Stafford developed a fluorogenic assay by replacing P1 and P3' residues of the substrate peptide with 2,4 dinitrophenyl-lysine and S-(N-[4-methyl-7-dimethylamino-coumarin-3-yl]-carboxamidomethyl)-cysteine, respectively. This enabled real-time activity measurements and made it possible to screen large number of compounds in a high-throughput assay. Boldt et al. synthesized the peptide SNAPtide for FRET-based assay that overcame the problem of photodegradation observed in the previous FRET substrate developed by Schmidt and Stafford, and optimized the assay conditions by including 0.01% w/v Tween 20 to stabilize BoNT/A LC and increase its protease activity [27]. The SNAPtide is a 13-amino acid long FRET peptide that uses a fluorescein isothiocyanate/4-((4-(dimethylamino)phenyl)azo) benzoic acid (FITC/DABCYL) FRET pair to produce a signal upon substrate cleavage. Feltrup and Singh further improved this assay by deriving the fluorescence internal quenching (FIQ) correction factors that allowed usage of this substrate over broader range of concentrations and temperatures [28]. Saunders et al. designed a multiplexed microsphere-based protease assay that uses high-throughput flow cytometry to screen for BoNT/A LC protease activity. They used the biotinylated and GFP tagged full-length substrate and several deletion mutants of it as controls to bind with streptavidin-coated microspheres. They demonstrated the use of this system by screening a library of 880 off patent drugs and bioavailable compounds and identifying ebsele as an *in vitro* inhibitor of BONT/A LC [29]. Salzameda et al. designed a 40 amino acid long FRET peptide substrate for BoNT/B LC protease activity for both in high throughput assays screening compound libraries and low throughput assays to determine kinetic parameters and modes of inhibition [30]. Rowe et al. have incorporated the use of ultra-performance liquid chromatography in measuring the BoNT protease activity, thus reducing the time required for resolving the substrate and product 10-fold, and also reducing the amount of solvent used by 28-fold [31]. They also improved the catalytic efficiency of BoNT/A by using the detergent Triton

X-100 in their reaction mixture. Recently, Mizanur et al. have tested the various substrates used in BoNT/A protease activity assays and determined that the full length SNAP25 protein gives the most consistent results. They also determined the ideal assay conditions for the enzyme activity that would be helpful in efficient screening of large compound libraries [32]. A detailed review of previous work on *in vitro* cell-based assays and *in vivo* assays for identification of BoNT inhibitors has been published earlier [33].

## 5.2 Substrate-based Inhibitors of BoNT/A

In the full length BoNT/A, the active site of the catalytic domain (LC) is covered by a 'belt' [34]. When the LC is released into the neuronal cytosol, the belt-binding region is exposed. SNAP-25, the substrate of BoNT/A protease activity wraps around the catalytic domain in a manner similar to the wrapping of the belt [35]. BoNT/A specifically cleaves a peptide bond in SNAP-25 between the residues Q197 and R198. A crystal structure of BoNT/A catalytic domain in complex with SNAP-25 peptide 197-QRATKM-202 provides a snapshot of key interactions between the substrate and the protein at the active site [36]. This and other structures of BoNT/A LC with substrate-based peptides provide a solid basis for substrate-based inhibitor design against BoNT/A.

The minimal cleavable segment of SNAP-25 for BoNT/A is DEANQ/RATK, where the scissile bond is located between Q197 and R198 [37]. And this provides opportunities for designing peptide and peptidomimetic inhibitors against BoNT/A that are smaller than this substrate and will be able to compete with SNAP-25 and are uncleavable. A number of peptide and peptidomimetic inhibitors have been designed and tested against BoNT/A and crystal structures of some of the enzyme-inhibitor complexes determined. Schmidt et al. identified the early substrate-based small peptide inhibitors of BoNT/A protease activity. Among the various peptide inhibitors tested, CRATKML was the most potent inhibitor with a  $K_i$  of 2  $\mu$ M [38]. Several modifications were introduced at the C-terminal of this peptide replacing the cysteine with a number of sulfhydryl-containing compounds, and 2-mercapto-3-phenylpropionyl was found to be the most effective replacement, improving the  $K_i$  to 330 nM (Table 1) [39]. These two studies also highlighted the importance of arginine at P1' position in the substrate-based inhibitors. Subsequently, the crystal structure of CRATKML in complex with BoNT/A LC was also determined providing the details of its interactions at the active site [40]. Further modifications in the peptide inhibitor CRATKML led to the development of a highly potent peptidomimetic inhibitor *DNP-DAB-RWT-DAB-ML* (where *DNP-DAB* is 4-(2,4-dinitrophenylamino)-2-amino-butanoic acid and *DAB* is 2,4-diaminobutanoic acid) with a  $K_i$  of 41 nM [41]. When compared to the apo structure, there are numerous structural changes observed around the active site in the crystal structure of the above peptidomimetic-enzyme complex. *DNP-DAB-RWT-DAB-ML* takes a helical shape in the active site compared to the extended structure of the substrate SNAP-25 (Fig. 2). Among the peptide inhibitors tested so far, a tetrapeptide seems to be the smallest peptide with good inhibitory activity. Peptides smaller than four amino acids showed drastic decrease in inhibitory potency [42]-[43]. These tetrapeptides correspond to the P1-P1'-P2'-P3' positions of the SNAP-25 substrate. The main features of a peptide inhibitor based on these studies appear to be: a zinc-chelating moiety, two positively charged moieties to engage negatively charged S1 and S1' pockets and a hydrophobic/aromatic moiety to fill the S3' hydrophobic pocket. Some of these tetrapeptides are also able to enter the neuronal cells without any adverse effect on the metabolic functions as measured by ATP production and P-38 phosphorylation [43].

### 5.3 Small molecule non-peptide inhibitors of BoNT/A

While a number of high affinity substrate-based peptide and peptidomimetic inhibitors have been identified for BoNT/A, there are certain drawbacks with such inhibitors e.g. their large size. Therefore, efforts are being made to identify small molecule non-peptide inhibitors that would meet better the criteria for a desirable drug. Boldt et al. synthesized a number of hydroxamate compounds using in situ chemistry and identified an active compound – *para*-chloro-cinnamic hydroxamate with an IC<sub>50</sub> value of 15 μM (Fig. 3). They further improved upon inhibitory activity with the synthesis of *ortho-para*-cinnamic hydroxamate that showed a K<sub>i</sub> of 0.3 μM [44]. Subsequently, the co-crystal structures of the two compounds in complex with BoNT/A catalytic domain were also determined, providing details of their interactions at the enzyme active-site [45]. Significant structural rearrangements affecting the electrostatic environment were observed at the S1' pocket. The hydroxamate moiety replaces the zinc-binding water molecule observed in the native structure and the hydroxyl oxygen of the hydroxamate moiety coordinates the catalytic zinc ion. The benzene ring is found nestled in a hydrophobic pocket towards the S1' site. This study provided valuable information on the plasticity of the BoNT/A LC active site. Inspired by the metalloprotease inhibitor drug captopril, a peptide-based inhibitor N-Ac-CRATKML and *ortho-para*-cinnamic hydroxamate, Moe et al. synthesized a number of mercaptoacetamide analogs of 5-amino-3-phenylpyrazole that showed low micromolar inhibition activities with the most potent one displaying an IC<sub>50</sub> of 4.8 μM [46]. Eubanks et al. screened a library of ~66,000 compounds (designed to target protein-protein interfaces) against recombinant BoNT/A LC, followed by cellular assay and *in vivo* murine toxicity bioassay [47]. One of the hits identified, NA-A1B2C10 inhibited the enzyme with an IC<sub>50</sub> of 12.5 μM, and showed no activity in cellular assays but was able to increase the time to death by 36% in murine toxicity assay at an injection dosage of 2.5 mM. Similarly, 2,4 dichlorocinnamic hydroxamic acid inhibits the recombinant enzyme with a K<sub>i</sub> of 0.3 μM, but showed no inhibition in cellular assays, but again was able to protect 16% mice from death by botulinum toxicity when injected at 1 mM concentration. Working on previous indole bis-amidine hits from the National Cancer Institute's Diversity set, Li et al. synthesized two different series of compounds and came up with a more potent inhibitor of BoNT/A protease activity with an IC<sub>50</sub> of 2.5 μM and highlighting the requirement of basic groups at the ends of the scaffold for potency [48, 49]. Opsenica et al. synthesized a number of 1,7-bis(alkylamino)diazachrysenes-based compounds that exhibited low micromolar IC<sub>50</sub> values against BoNT/A LC [50]. Interestingly, these compounds inhibited other targets in the malaria parasite *P. falciparum* with nanomolar IC<sub>50</sub> values and Ebola virus with IC<sub>50</sub> values in low micromolar range [51],[50]. Videnovic et al. synthesized a second generation of 4-amino-7-chloroquinoline based inhibitors of BoNT/A LC with K<sub>i</sub> values improving up to 103 nM and 89% protection of SNAP-25 at 30 μM inhibitor concentration in primary neurons [52]. Cai et al. used a two-tiered assay for screening a small molecule library of 16,544 compounds identifying nine active compounds with a K<sub>i</sub> of 9 μM for their most potent inhibitor that also showed inhibition in cellular assays with an IC<sub>50</sub> of 47 μM and no apparent toxicity [53]. Thompson et al. designed and tested a number of diverse compounds with hydroxamic acid as the zinc chelating group, their most potent compound PT1 showed an IC<sub>50</sub> of 4.6 μM. They determined the co-crystal structures of these inhibitors in complex with BoNT/A that showed significant movement in the 60/70 loop and the 360/370 loop upon binding of these inhibitors implicating an induced fit mechanism [54]. Ruthel et al. tested a number of analogues of their previously identified inhibitor – NSC104999 from

NCI's open repository and identified an inhibitor - NSC95654 with a  $K_i$  of 1.8  $\mu\text{M}$  against purified enzyme and substantial inhibitory activity within chick motor neuron cells as well [55]. Silhar et al. synthesized 1-adamantylacetohydroxamic acid that showed submicromolar potency against purified enzyme but no protection against BoNT/A induced cleavage of SNAP-25 in neuronal cells. So, they synthesized dioxazole-based and carbamate-like prodrugs of the inhibitor. The carbamate prodrugs proved successful and their most potent compound in this category, a benzylcarbamoyl hydroxamate displayed inhibition activity with an  $\text{EC}_{50}$  of 20  $\mu\text{M}$  in cells [56]. Caglic et al. tested a number of quinolinols and hydroxyquinolines against BoNT/A LC using the SNAPtide assay and identified a number of actives, the best one with an  $\text{IC}_{50}$  of 0.8  $\mu\text{M}$ . While most of the compounds showed low solubility at neutral pH, it improved at low pH of 1.0 suggesting that these could be good candidates for oral dosage. Various other ADME parameters were also found to be favorable [57].

#### 5.4 Covalent inhibitors of BoNT/A

Although numerous covalent drugs have been successfully used against diseases like cancer, cardiovascular diseases, CNS related diseases and bacterial infections, yet owing to safety concerns, they are rarely considered when starting a target-based drug discovery project [58]. Recently, a couple of covalent inhibitors of BoNT/A have been reported that opens this avenue for anti-botulism drug discovery. These inhibitors, MTSEA ((2-aminoethyl) methanethiosulfonate) and MTSPA (3-aminopropyl methanethiosulfonate hydrobromide) attack Cyst165 (that lies in close vicinity of the catalytic zinc) and covalently modify its side chain. The better of the two inhibits BoNT/A protease activity with a  $K_i$  of 7.7  $\mu\text{M}$  that improves further with longer incubation of the inhibitor with the enzyme [59].

### 6. INHIBITORS OF BoNT/B

After BoNT serotype A, serotype B is the most frequently diagnosed serotype responsible for botulism in humans. In addition to BoNT/A, BoNT/B also is used for therapeutic and cosmetic purposes. Compared to BoNT/A, it has received relatively lesser attention from a drug discovery point of view. BoNT/B specifically cleaves the peptide bond between residues Gln76 and Phe77 of the substrate protein – VAMP [60, 61]. A small molecule, 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin (ICD 1578) was identified as one of the earliest inhibitors of BoNT/B with an  $\text{IC}_{50}$  of 27.6  $\mu\text{M}$  based on its similarity with an analogue of phosphoramidon which is known to be a metalloprotease inhibitor (Fig. 4) [62]. Anne et al. targeted the S1, S1' and S2' subsites in the active site and synthesized libraries of pseudotripeptides containing beta-amino thiols. They then replaced the amino acids of the pseudotripeptides with non-natural amino acids. The most potent inhibitor showed a  $K_i$  of 20 nM [63]. They further enhanced the inhibition by replacing the thiol functionality with symmetric disulfides, improving the  $K_i$  to 3.4 nM [64].

### 7. INHIBITORS OF BoNT/E

Among the three serotypes (A, B and E) that affect humans, BoNT/E is the fastest acting BoNT. But very little work has been done so far in terms of drug discovery against this serotype. BoNT/E specifically cleaves the peptide bond between Arg180 and Ile181 residues of the substrate protein SNAP-25 [65]. A substrate-based peptide inhibitor RIME has been identified and its co-crystal structure in complex with BoNT/E determined. It inhibits BoNT/E protease activity with a  $K_i$  of 69  $\mu\text{M}$  (Fig. 4) [66]. The co-crystal structure provides insights into protein-

ligand interactions at the BoNT/E active site and opens an avenue for structure-based drug discovery. The peptide RIME represents the substrate sequence from residue 180 to 183 of SNAP-25, spanning the scissile bond. It escapes cleavage by BoNT/E but has enough affinity to bind at the active site to act as an inhibitor. It makes numerous hydrogen bonding and hydrophobic interactions at the active site. The P1 residue, Arg180 displaces the nucleophilic water observed in the native structure and its carbonyl oxygen co-ordinates the catalytic zinc. Its side chain guanidinium group forms a salt bridge with E158. The P1' residue Ile181 goes into the hydrophobic S1' subsite, which is created by residues T159, F191 and T208 (three letter and single letter codes are used for amino acids of substrate and enzyme, respectively). The hydrophobic side chain of P2' residue Met182 is also accommodated in a hydrophobic pocket created by F191, Y354 and Y356. The P3' residue Glu183 interacts with the flexible 250 loop, thus stabilizing it. These details provide ample opportunities for designing better peptide as well as non-peptide small molecule inhibitors. The first small molecule non-peptide inhibitor of BoNT/E was identified through a structure-based virtual screening of the diversity set I of NCI compound repository that has 1990 diverse molecules representative of all the ~260,000 compounds. The molecules from the diversity were docked against BoNT/E protease domain using docking program AutoDock and 18 compounds were shortlisted for HPLC-based inhibition assay. A number of compounds were found active at 250  $\mu\text{M}$  with the most potent one (NSC77053 or (2-(9H-fluoren-2-ylcarbonyl)benzoic acid)) showing a  $K_i$  of 1.29  $\mu\text{M}$  [67]. A comparison of the co-crystal structure of RIME-BoNT/E complex and the docked compound NSC77053 showed that many interactions observed in enzyme-peptide complex are also observed in the enzyme-small molecule complex. The carboxy group of the benzoic acid moiety of NSC77053 coordinated the catalytic zinc in a similar manner as the carbonyl oxygen of the peptide as described earlier and the benzene ring nestled in the S1' pocket similar to the hydrophobic side chain of Ile181. The hydrophobic/aromatic fluorene moiety goes into the same pocket as the hydrophobic side chain of Met182. These are the pharmacophore features that could be exploited in developing new BoNT/E inhibitors.

## 8. INHIBITORS TARGETING EXOSITES ON BoNTs

The SNARE assembly protein, SNAP-25 wraps around BoNT/A LC making an extensive network of interactions similar to the belt region that wraps around the catalytic domain in case of full length BoNT/A (Fig. 1). In this BoNT/A-SNAP-25 complex, the N-terminal residues of SNAP-25 (residues 147-167) form an  $\alpha$ -helix and interact on the rear side of BoNT/A, the C-terminal residues (201-204) form a distorted  $\beta$ -strand and the spanning residues are random coil [35]. The regions on BoNT/A where these secondary structure elements bind, have been termed  $\alpha$ - and  $\beta$ -exosites, respectively. These interactions between BoNT/A and SNAP-25 have been found to be critical in substrate recognition and cleavage [37]. Hence they also could be potentially used as targets for designing inhibitors of *in vivo* BoNT/A protease activity. SA couple of small molecule natural product inhibitors have been identified for BoNT/A and BoNT/B that target these exosites located away from the zinc dependent active site. Silhar et al. tested the main components of Echinacea plant (a Native American medicinal plant used widely over centuries to treat infections and wounds) that are phenolic caffeoyl derivatives, namely D-chicoric acid, caftaric acid and chlorogenic acid. The most potent of these natural products, D-Chicoric acid, inhibited BoNT/A activity with an inhibition constant of 0.7  $\mu\text{M}$  and improved the overall inhibition of BoNT/A activity in a synergistic manner along with an active site inhibitor, like the *ortho-para*-cinnamic hydroxamate (Fig. 5) [68]. Enzyme kinetic analysis revealed that

D-Chicoric acid binds to an exosite and displays noncompetitive partial inhibition. Eubanks et al. used a high-throughput screening approach to test the Johns Hopkins Clinical Compound Library composed of over 1,500 existing drugs and identified Lomofungin, a natural product, showing noncompetitive inhibition with a  $K_i$  of 6.7  $\mu\text{M}$ . Kinetic data indicated that this inhibitor could possibly bind at the  $\beta$ -exosite [69]. L-Chicoric acid was found to inhibit BoNT/B protease activity with an  $\text{IC}_{50}$  value of 7.5  $\mu\text{M}$  through a similar mechanism observed in the case of BoNT/A, by binding far away from the active site in a region where the SNARE motif of substrate peptide interacts with the protease enzyme [70]. Recently, Hu et al. conducted molecular dynamics simulations, applying replica-exchange MD (REMD) simulations for better sampling of large interaction interface, to explore the binding modes of D-Chicoric acid and Lomofungin on BoNT/A and rationalized the previous experimental findings as discussed above. They also conducted virtual screening of MLSMR library consisting of  $\sim 350,000$  drug-like molecules, combining ligand-based similarity search and structure-based docking. They tested 167 virtual hits in a FRET-based assay and identified 8 active compounds, the most potent of which showing a  $K_i$  of 90 nM [71].

## 9. CONCLUSION AND FUTURE DIRECTIONS

BoNTs not only pose a bioterror and biowarfare threat, their rapidly increasing use as therapeutic and cosmetic agent necessitates that we have drugs at our disposal even in case of an accidental overdose to reverse the symptoms. Significant progress has been made towards antibody-based therapy and vaccination for at-risk populations. But, the post-intoxication therapeutic intervention remains elusive. A tremendous amount of work has been done to decipher the structure of BoNTs and their mechanism of action. Determination of crystal structures of the protease domain has enabled virtual screening of large libraries of drug-like molecules. Newly designed high-throughput screening assays have also reduced the turn over time for identifying active compounds. Drug discovery projects are being targeted against the BoNT catalytic domain for identifying substrate-based peptide inhibitors, peptidomimetics, and SMNPIs. As a result of these efforts, a number of potent peptide-based inhibitors and SMNPIs have been identified and important pharmacophore features required for BoNT inhibition are emerging. Zinc-chelating feature seems to be the most important across various serotypes as discussed in the case of serotype A, B and E. Around this anchor feature, other features e.g. requirement for positively charged groups emerged from the peptide-based inhibitors for BoNT/A. Although this feature was observed in many small molecule inhibitors too, especially hydroxamate inhibitors, it appears that a hydrophobic feature (e.g. the chloro-benzene or the adamantane group) is also required for better activity. Along with the zinc-chelating feature, the hydrophobic feature also seems to be a common requirement among the three serotypes, because this feature has been found wanted for serotype B (e.g. in pseudotriptide inhibitors) and serotype E (the fluorene or phenyl moiety in NSC77053).

There are a few challenges in developing a structure-activity relationship for the inhibitors of a given serotype, and more so in the case of pan-active inhibitors. While the core of the protease is relatively rigid, the superficial structure is highly dynamic, there are flexible loops and induced-fitting occurs between the enzyme and the inhibitor. This also brings in an added complexity for virtual screening protocols, as most of them use rigid receptor. Integrating loop flexibilities in a virtual screening protocol would be very difficult because of intensive computing power required to do so. And when it comes to virtual screening by docking,

optimization of force-field parameters for the zinc ion in the BoNT environment may also help in correctly predicting the binding modes and the binding energies of the inhibitors. Inclusion of conserved water molecules as part of the receptor may also help in improving the hit rate.

Another aspect of BoNT inhibition is that the site of substrate recognition located away from the active site. The inhibitors found active in assays using purified light chain with partial SNAP-25 used as substrate may not work against the full-length substrate in the neuronal cytosol. While it brings a little doubt about the effectiveness of active site based inhibitors, it also provides new opportunities for targeting the enzyme in ways. Indeed, a few inhibitors have been identified that do not bind at the zinc-centric active site and inhibit the enzyme in a noncompetitive manner. These inhibitors are predicted to target the exosites and hold potential. Also, disconnect exists between the inhibition of protease activity of purified light chain by inhibitors and their efficacy in cell-based assays and animal toxicity models [47, 72]. As more and more inhibitors with potencies approaching low and sub-nanomolar values emerge, the subsequent issues about cell permeability, metabolic fate and ADME issues will come to the forefront.

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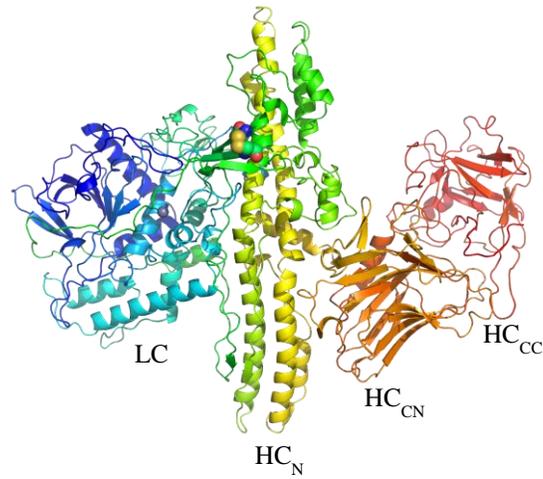
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**Figure 1:** Domain organization in BoNT/A. The amino acid chain represented in ribbons is colored in rainbow colors starting from N-terminal as blue and C-terminal as red. LC is the catalytic domain, HC<sub>N</sub> is the translocation domain, HC<sub>CN</sub> and HC<sub>CC</sub> make the receptor-binding domain. Zinc is shown as grey ball in LC and disulfide bond between LC and HC<sub>N</sub> is also shown as balls in atomic colors. The figure was made using the crystal structure deposited in PDB with ID: 3BTA.

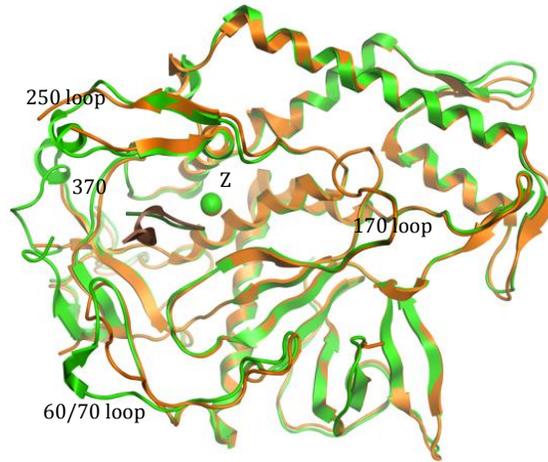


Figure 2. Peptide inhibitors of BoNT/A. Cartoon diagram of BoNT/A-RRGF complex superimposed (PDB ID: 3QW5) in green color with BoNT/A-DNP-DAB-RWT-DAB-ML (PDB ID: 3DS9) in brown color. Zinc is shown as sphere, RRGF is shown in dark green and DNP-DAB-RWT-DAB-ML in dark brown.

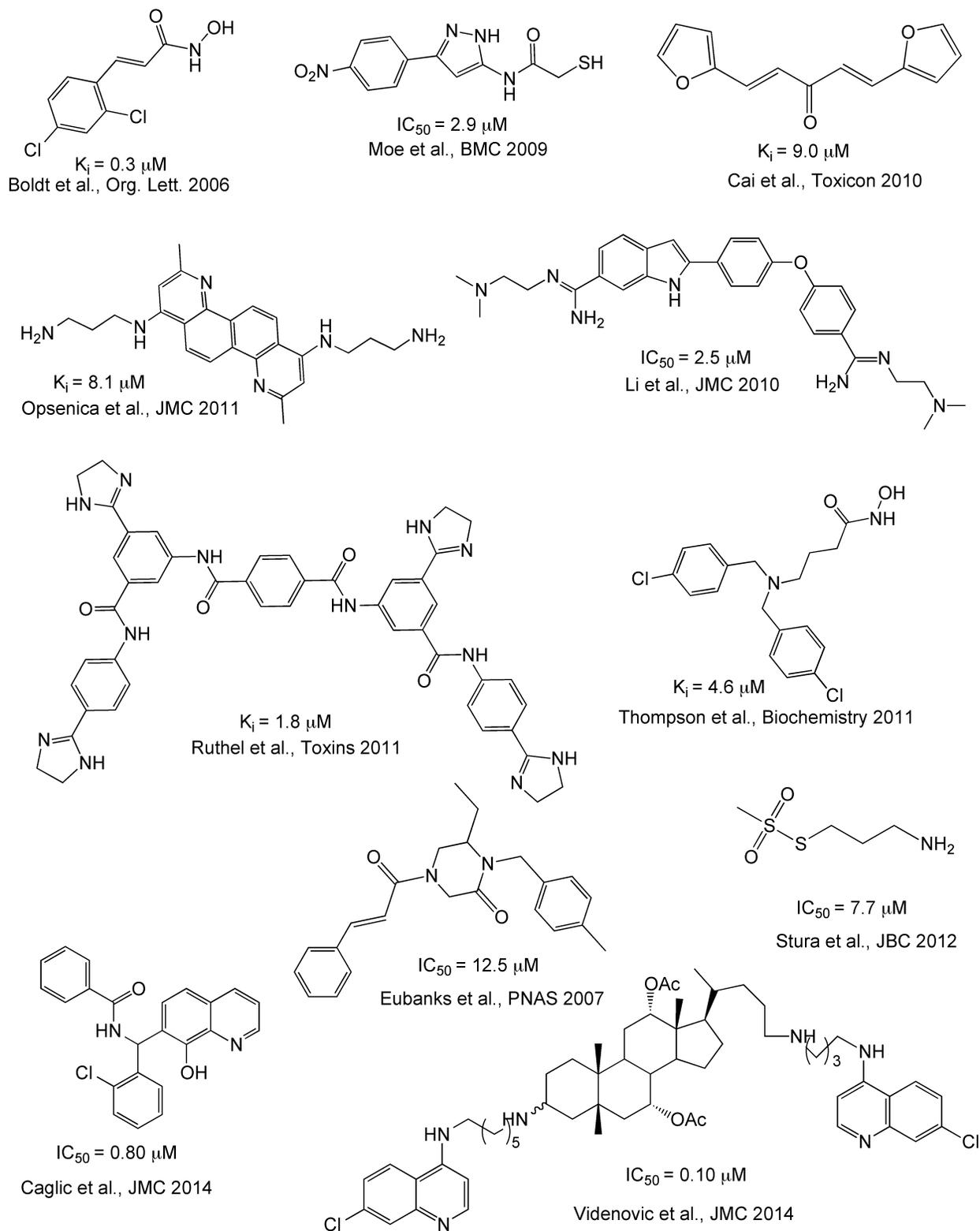
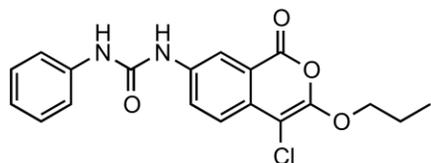
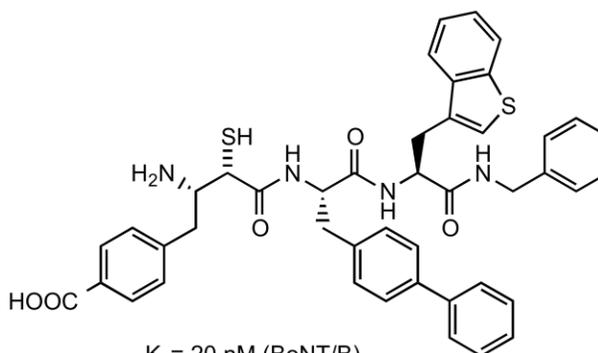


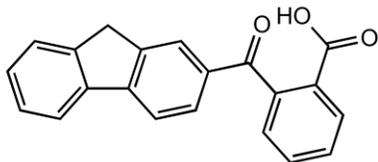
Figure 3. BoNT/A Inhibitors with their respective inhibitory potencies against purified enzyme (catalytic domain).



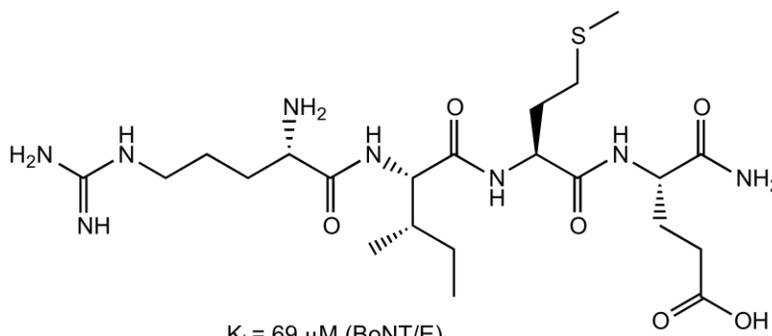
$IC_{50} = 27.6 \mu\text{M}$  (BoNT/B)  
Adler et al. FEBS Letters 1998



$K_i = 20 \text{ nM}$  (BoNT/B)  
Anne et al. JMC 2003

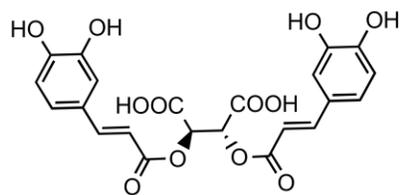


$K_i = 1.29 \mu\text{M}$  (BoNT/E)  
Kumar et al., Chem Comm 2012

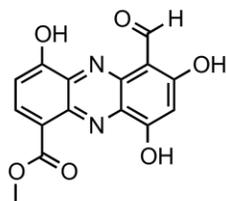


$K_i = 69 \mu\text{M}$  (BoNT/E)  
Agarwal et al., JBC 2008

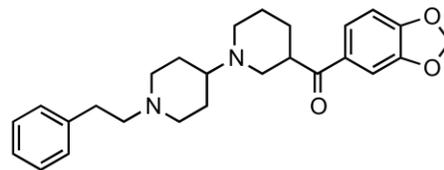
Figure 4. Inhibitors of BoNT/B and BoNT/E with their respective inhibitory potencies.



D-Chicoric Acid  
 $K_i = 0.7 \mu\text{M}$   
Silhar et al. JACS 2010



Lomofungin  
 $K_i = 6.7 \mu\text{M}$   
Eubanks et al. ACS MCL 2010



Inhibitor C2  
 $K_i = 0.09 \mu\text{M}$   
Hu et al. JCAMD 2014

Figure 5. Exosite inhibitors of BoNT/A with their respective inhibitory potencies.

**Table 1:** Peptide and Peptidomimetic inhibitors of BoNT/A endopeptidase activity

Peptide/ Peptidomimetic	Inhibition ( $\mu\text{M}$ )		PDB id	References
	IC <sub>50</sub>	K <sub>I</sub>		
RRGC	1.5	0.157	3C88	[73]
RRGM		0.845	3C89	[73]
RRGL		0.660	3C8A	[73]
RRGI		0.786	3C8B	[73]
QRATKM	133		3DDA	[36]
RRATKM	95		3DDB	[36]
RRGF	0.9		3QW5	[42]
CRGF	1.5		3QW8	[42]
RRFC	1.8		3QW7	[42]
RRYC	5.4			[42]
CRGC	8.0			[42]
WRGC	10.0			[42]
QRGC	14.0			[42]
CRRGC	43.0			[42]
RRGCM	26.1			[42]
RRKRL	28.7			[42]
N-Ac-CRATKML		2.0	3BOO	[39, 40]
I1 ( <i>DNP-DAB-RWT-DAB-ML</i> )		0.041	3DS9	[41]
I2		6.5		[41]
I6		8.3		[41]
I7		3.3		[41]
I8		0.98		[41]
I9		0.094		[41]
I10		0.05		[41]
I11		0.32		[41]
I12		0.1		[41]
I13		0.39		[41]
Compd: JTH-NB72-35		0.314		[74]
Compd: JTH-NB72-38		0.990		[74]
Compd: JTH-NB72-39		0.638	3NF3	[74]