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Structural Characterization of Three Novel Hydroxamate-based Zinc Chelating Inhibitors of the *C. botulinum* Serotype A Neurotoxin Light Chain Metalloprotease Reveals a Compact Binding Site Resulting from 60/70 Loop Flexibility†

Aaron A. Thompson¹, Guan-Sheng Jiao², Seongjin Kim², April Thai², Lynne Cregar-Hernandez³, Stephen A. Margosiak³, Alan T. Johnson², Gye Won Han¹, Sean O'Malley², and Raymond C. Stevens^{1,*}

¹ Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

² Department of Chemistry, PanThera Biopharma LLC, Aiea, HI 96701

³ Department of Lead Discovery, PanThera Biopharma LLC, Aiea, HI 96701

Abstract

Neurotoxins synthesized by *Clostridium botulinum* bacteria (BoNT), the etiological agent of human botulism, are extremely toxic proteins making them high-risk agents for bioterrorism. Small molecule inhibitor development has been focused on the light chain zinc-dependent metalloprotease domain of the neurotoxin, an effort that has been hampered by its relatively flexible active site. Developed in concert with structure-activity relationship studies, the X-ray crystal structures of the complex of BoNT serotype A light chain (BoNT/A LC) with three different micromolar potency hydroxamate-based inhibitors are reported here. Comparison with an unliganded BoNT/A LC structure reveals significant changes in the active site as a result of binding by the unique inhibitor scaffolds. The 60/70 loop at the opening of the active site pocket undergoes the largest conformational change, presumably through an induced-fit mechanism, resulting in the most compact catalytic pocket observed in all known BoNT/A LC structures.

Neurotoxins synthesized by the anaerobic bacterium *Clostridium botulinum*, the causative agents of botulism, are categorized into seven distinct serotypes based on immunogenic properties (A–G). These toxins typically enter peripheral cholinergic neurons where they inhibit release of the neurotransmitter acetylcholine by preventing synaptic vesicle docking with the presynaptic membrane, ultimately resulting in flaccid muscle paralysis of the host. Human botulism is caused by the *C. botulinum* neurotoxin (BoNT) serotypes A, B, E, and F, with the most potent of these, type A (BoNT/A), displaying a lethal dose of only 1 ng kg^{−1} of body weight (1, 2). Besides causing naturally occurring botulism, BoNT is also classified

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*Address correspondence to: The Scripps Research Institute, 10550 North Torrey Pines Rd., GAC-1200, La Jolla, CA 92037. Tel.: 858-784-9416; Fax: 858-784-9483; stevens@scripps.edu.

SUPPORTING INFORMATION AVAILABLE

Additional figures showing a structural comparison of the active site pockets between BoNT/A LC and thermolysin; electron density maps of the inhibitor bound and unliganded active site pockets; structural comparison with the SNAP-25 peptide from PDB code 3DDA; 60/70 loop proximity with the LC C-terminus. This material is available free of charge via the Internet at <http://pubs.acs.org>.

by the Centers for Disease Control and Prevention as a Category A agent making it one of the highest-risk threat agents for bioterrorism (<http://emergency.cdc.gov/agent/agentlist-category.asp>). Current treatment regimens rely on antibody-based antitoxins that cannot interact with the toxin once it has entered the neuron, where the activity of the BoNT/A light chain can persist for months (reviewed in (3)). The development of small molecule inhibitors as intraneuronal therapeutics is a crucial unmet need.

The crystal structure of BoNT shows three functional domains comprised of a ~50 kDa light chain (LC) and a heavy chain (HC) containing two segments that are each approximately ~50 kDa (Figure 1A,B (4)). The C-terminal portion of the HC is the cell binding domain and is responsible for docking the toxin to gangliosides and a protein receptor(s) on presynaptic neurons resulting in toxin endocytosis. The translocation domain, at the N-terminal portion of the HC, mediates escape of the toxin LC from the endosome into the neuronal cytosol. The LC is a zinc-dependent endopeptidase that is involved in serotype-dependent cleavage of one or more members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex of proteins involved in synaptic vesicle docking. BoNT/A specifically cleaves between residues Gln197 and Arg198 of the 206 residue SNAP-25 protein (synaptosome-associated protein of 25 kDa). A unique feature of BoNT LCs is their requirement for relatively long substrates when compared to other zinc-dependent endopeptidases outside of the clostridial family. BoNT/A LC requires a minimal SNAP-25 peptide sequence of ~51 amino acids to achieve efficient cleavage, and optimal binding occurs with only the full length SNAP-25 (5, 6). The crystal structure of SNAP-25₁₄₁₋₂₀₄ (residues 141–204) bound to BoNT/A LC (residues 2–420 with active site mutations E224Q, Y366F) provides an explanation for this finding as binding involves protein exosites that anchor the substrate and position the scissile bond for cleavage (7). Binding of SNAREs to protein exosites flanking the active site pocket likely influences the high degree of substrate specificity because of the decrease in entropy which enables the scissile bond to orient into the deep active site via a sharp stereochemically strained conformation (Supplemental Figure S1A,B). Insight into SNAP-25 - BoNT/A LC subsite interactions were elucidated by the BoNT/A LC structure (native active site) complexed with a smaller SNAP-25₁₉₇₋₂₀₂ peptide fragment (residues 197–202) (8). Catalysis occurs within a relatively deep active site pocket measuring ~22 by ~22 Å wide and ~25 Å deep. Cleavage by LCs of substrate SNARE proteins does not allow for the formation of ternary complexes that are believed to drive synaptic vesicle fusion and exocytosis of neurotransmitters (reviewed in (9–13)).

The clostridial neurotoxin LCs are zinc-dependent endopeptidases representing a unique family of zinc metalloproteases in the gluzincin superfamily with thermolysin being the prototypical member. The primary sequence of thermolysin and the BoNT LCs contain a conserved HEXXH motif that is required for chelation of a catalytic zinc ion and a putative interaction with a water molecule thought to be involved in the general base mechanism of catalysis (4, 7). While a structural based homology server (Dali; (14)) search suggests thermolysin is currently the closest structural neighbor to clostridial LCs, comparison of the solvent accessible surface of the active site pockets reveals significant differences in the shape and volume of these pockets (Supplemental Figure S1). In the serotype A LC, His223 and His227 from the HEXXH motif are involved in binding zinc along with a conserved Glu262 that is located approximately thirty amino acids downstream of the motif (Supplemental Figure S1E, S1F). The Glu224 from this conserved HEXXH motif is believed to act as a general base by polarizing a water molecule, thereby preparing it for nucleophilic attack of the scissile peptide bond's carbonyl carbon atom. The primary sphere of residues coordinating the catalytic zinc is identical among clostridial neurotoxin LCs and other members of the gluzincin family, such as the prototypical thermolysin protease; however, there are potentially significant differences in the secondary sphere of residues that

can be attributed to diversity in substrate preference. Differences include the Arg363 and Tyr366 of BoNT/A LC which are believed to stabilize the nascent oxyanion formed during the proteolytic cleavage event, residues lining the S1, S1', S2', and S3' binding pockets, and the surface area of active site pockets (Supplemental Figure S1). These differences from other gluzincin proteases suggest that development of inhibitors selective for zinc-dependent endopeptidases from the *Clostridium* family is possible.

There are several reports of small molecule inhibitors of BoNT/A LC. Most of these inhibitors bear a zinc binding group, either a hydroxamic acid (15, 16), a thioacetyl group (17), or a hydroxyquinoline group (18); a subset are highly positively charged but lack a clear zinc binding group (19). PanThera Biopharma has embarked on a program to develop postexposure intraneuronal therapeutics for BoNT/A intoxication through the optimization of hydroxamic acid inhibitors; multiple distinct structural series are currently under investigation. In this study, we present the X-ray crystal structures of inhibitors representing three novel branched distinct scaffolds and discuss significant interactions between the inhibitors and their BoNT/A LC target, focusing on the key 60/70 loop (BoNT/A LC residues 60–79) movement which appears to be critically important to understanding the binding of these inhibitors.

Experimental Procedures (Materials and Methods)

BoNT/A LC Cloning, Expression and Purification

The cDNA encoding a 424 amino acid gene for the BoNT/A LC protease was synthesized by DNA2.0 with codon optimization for expression in *E. coli* and then cloned into the inducible T7-based expression vector pET23a (Novagen). After expression in *E. coli* BL-21 (DE3) pLysS, the cells were resuspended in 20 mM TRIS [pH 8.0], 300 mM NaCl, 10 mM imidazole [pH 8.0] and lysed using a cell disruptor (Microfluidics). The lysate was centrifuged at $20,000 \times g$ and the supernatant filtered with a 0.2 μ m cutoff syringe filter. The lysate was then transferred to a gravity column containing 5 mL bed volume of IMAC sepharose 6 FF (GE Healthcare) charged with $ZnCl_2$ and equilibrated with lysis buffer, and eluted with a linear gradient from 10 mM to 400 mM imidazole in 20 mM TRIS [pH 8.0] and 300 mM NaCl. Fractions containing BoNT/A LC were pooled and ammonium sulfate was added to a final concentration of 0.8 M. This protein was loaded onto a 5 mL Butyl-FF column (GE healthcare) and eluted using a linear gradient from 0.8 to 0.1 M ammonium sulfate in 50 mM HEPES [pH 7.5]. The fractions containing BoNT/A LC were again pooled and concentrated using Amicon Ultra-4 30k MWCO centrifugal filtration devices (Millipore) and then run over a Superdex 200 column equilibrated in 200 mM NaCl and 10 mM HEPES [pH 7.5]. The purified protein was concentrated to ~15 mg/mL for crystallization.

BoNT/A LC Proteolytic Assay

A fluorescence resonance energy transfer (FRET) assay was used to determine the inhibition kinetics and K_i values of BoNT/A LC inhibitors using Abz-TdRIDEANQRATKnLK(dnp)-NH₂ as the peptide substrate. Cleavage was measured as an increase in fluorescence with λ_{ex} = 320 nm, λ_{em} = 420 nm. The 100 μ L reaction contained 13 nM BoNT/A LC₁₋₄₂₉ in 30 mM HEPES [pH 7.3], 0.05 mM ZnAcetate, 0.05% Tween 20, 0.1 mg/mL BSA, 2.5% DMSO and 25 μ M peptide substrate. The reaction was initiated with substrate addition and incubated 10 minutes (23 °C) while measuring the increase in fluorescence to obtain a steady state rate of product formation. Steady state rates were used to determine K_i values by fitting to the classical competitive inhibition equation.

Crystallization and Structure Determination

Crystals are grown by sitting nanodrop vapor diffusion at 20 °C using ~7–10 mg/mL protein sample, 0.5 mM inhibitor compound when applicable, and using the Innovadyne Screenmaker crystallization robot. Zinc-bound BoNT/A LC and the PanThera compound 2 (PT-2; Figure 1C) BoNT/A LC complex belonging to the spacegroup $P2_1$ and $P2_12_12$ respectively, grew in ~10 days from 5% glycerol and 15% PEG 6000, with glycerol added to a final concentration of 20% for cryoprotection. The PanThera compound 3 (PT-3; Figure 1C) BoNT/A LC complex crystals grew in ~30 days with a precipitant/well solution containing 10% PEG 6000, 100 mM MES [pH 6.5], and the PanThera compound 1 (PT-1; Figure 1C) BoNT/A LC complex crystals grew in ~30 days with a precipitant/well solution containing 13% PEG 6000, 100 mM MES [pH 6.9]. Ethylene glycol was briefly added to a final concentration of 20% for cryoprotection. Diffraction data were collected at the Advanced Light Source beamlines 12.3.1 and 4.2.2 (Berkeley, CA) and SSRL beamline 11.1 (Palo Alto, CA). Reflections were processed using HKL2000 (20), and the initial structure solution was obtained by molecular replacement with Phaser (21) using BoNT/A LC (PDB code 3DDA (8)) as the search model. The PT-1 complexed crystals displayed anisotropic diffraction; a complete dataset was obtained by merging data collected from two crystals. Manual model rebuilding was performed using Coot (22) and refined with the Phenix software suite (23), and the PT-1 structure was refined further using autoBUSTER (24). TLS refinement was used at the final stages of refinement with three TLS domains defined per protease molecule (25). Figures were generated with Pymol Molecular Graphics System version 1.0r2 and structure-based alignments were performed using Pymol's "super" algorithm (www.pymol.org). Data and refinement statistics are listed in Table 2.

Coordinates and Structure Factors

The coordinates and structure factors have been deposited in the RCSB Protein Data Bank under the accession codes 3QIX (unliganded), 3QIY (PT-1 complex), 3QIZ (PT-2 complex) and 3QJ0 (PT-3 complex).

Inhibitor Synthesis

Synthesis of the inhibitors will be described elsewhere (manuscripts in preparation). PT-2 was prepared according to previously reported methods for structurally related Anthrax Lethal Factor inhibitors (26).

RESULTS

BoNT/A LC Structures Bound with Zinc

Using a construct consisting of the first 424 residues of BoNT/A LC, we obtained the structure of its ligand-free Zinc-bound form with crystals belonging to the $P2_1$ spacegroup. This crystal structure contained two molecules in the asymmetric unit; the individual molecules were not significantly different from each other with a C_α RMSD of 0.38 Å. Several loops located within close proximity of the large active site opening, the 250 and 190 loops, display relatively weak or absent electron density in both LC molecules suggesting flexibility in this region. Electron density for a single zinc ion was observed in each catalytic site coordinated by His223, His227, Glu262, and a water molecule with weak density is present in the absence of inhibitor (Supplemental Figure S2).

The three inhibitors used in this study are shown in Figure 1C. Each contains three binding moieties, two halogenated phenyl rings and a zinc-binding hydroxamate group, which are connected via three different branched scaffolds. Each ring lies at the end of a flexible arm with one arm binding to the S1' site (referred here as S1' arm) and the other to the S2' and S3' sites (referred here as S3' arm). These inhibitors display K_i values in the single digit μM

range (Table 1). Despite the presence of relatively large solvent channels leading to the active site pockets, crystallization of BoNT/A LC complexed with each of the three inhibitors required cocrystallization; attempts at soaking them into the P2₁ BoNT/A LC crystals were unsuccessful (Table 2). Interestingly, the inhibitor-bound structures crystallized in a different space group, P2₁2₁2, with significantly different cell dimensions. The ligand binding sites for the three inhibitor complexes in these crystal structures are shown in Figure 2 and Supplemental Figure S2.

Ligand-binding Conformational Changes Indicate Possible Induced Fit

The inhibitor-bound structures confirm that the hydroxamate moiety is engaged in a planar bidentate interaction with the catalytic zinc (Figure 2). The hydroxamate groups appear to be locked in place by hydrogen bonding interactions with the backbone carbonyl of Phe163, and the catalytically important residues Glu224 and Tyr366 (Figure 2). The latter is believed to play a role in the stabilization of the oxyanion formed during native substrate cleavage. While the arms of these inhibitors have been designed to exploit the relatively hydrophobic nature of the active site pocket, the scaffolds used in PT-2 and -3 have additional groups that facilitate potential hydrogen bonding with the “roof” of the catalytic pocket. PT-2 contains a methoxy group that interacts with the backbone of residues 161 and 163 through a water mediated hydrogen bond, while the sulfur dioxide group of PT-3 interacts directly with the backbone nitrogen of residue 163. A structure-based superposition of the BoNT/A LC structures that down-weights amino acid pairings with high relative variability has revealed multiple conformational changes that are presumably a consequence of inhibitor binding (Figure 3). The most significant differences occur around Phe194, the 360/370 loop (residues 366–372), and the 60/70 loop (residues 61–79).

Phe194Can Assume a Different Rotamer on Ligand Binding

Aromatic groups from the S1' arm of the inhibitors exploit the deep cavity of the S1' subsite of the binding pocket which in the SNAP-25_{197–202} peptide-bound structure (8) is formed by Phe194 and Asp370. Phe194 appears poised to facilitate cation- π stacking interactions with the guanidinium group of the SNAP-25 P1' arginine residue, and Asp370 forms the “floor” of the S1' subsite via formation of a salt bridge with the P1' arginine (Supplemental Figure S3). The positioning of Phe194 and Asp370 from the SNAP-25_{197–202} bound structures is almost identical to that of the BoNT/A holotoxin structures (PDB codes 3BTA, 2NYY, 2NZ9), suggesting these residues are prepositioned for optimal interaction with the P1' residue. This is supported by the fact that Phe194 and Asp370 are also pre-positioned in almost all previously published apo and zinc loaded BoNT/A LC structures (PDB codes 1XTF, 2IMC, 2ISE, 2ISH, 2W2D, 3BOK, 3BON, 3BWI). Surprisingly, Phe194 from the zinc bound P2₁ structure presented in this study is observed in the p90 rotamer configuration (rotamers described in (27)), and Asp370 from a separate zinc bound LC structure (PDB code 2ISG) is pointed “Out” and away from the catalytic center, thereby alluding to the structural plasticity of the active site pocket (Table 3, Supplemental Figure S2).

The S1' arms of the inhibitor structures all reveal hydrophobic stacking against the sidechain of Phe194 which is enabled by assuming alternate sidechain rotamer conformations with negligible deviations to the backbone (Figure 3B). Residue Phe194 from the PT-1 bound BoNT/A LC structure adopts the m-85 rotamer conformation similar to the holotoxin, and SNAP-25- and most apo/zinc-bound BoNT/A LC structures. Phe194's sidechain conformation in the structures of PT-2 and -3 complexes are rotated to the less commonly observed p90 rotamer optimizing the π -stacking interactions with these inhibitors (Figures 2 & 3B, Supplemental Figure S2). When compared with a random PDB dataset containing 1570 occurrences of Phe at 1.7 Å resolution or higher, 44% of the structures adopt the m-85 rotamer versus 13% for the p90 rotamer (27).

Conformational Changes to the 360/370 Loop Upon Ligand Binding: Asn368 or Phe369 can replace Asp370 in forming the S1' subsite

The conformational flexibility of this domain has been previously described with small molecule (28) and peptidyl ligands (29). Interactions between the inhibitor hydroxamate group's carbonyl oxygen and the exocyclic hydroxyl group of Tyr366, in combination with hydrophobic and van der Waals forces between the S3' arms of the compounds, appear to "pull" this amino acid residue into the active site, thereby altering the positioning of the 360 loop (Figure 3B,C). Binding by PT-3 results in the most dramatic changes of this loop with residues Asn368 and Phe369 showing significant deviations from the zinc bound BoNT/A LC; the C $_{\alpha}$ of Asn368 is displaced by 3.0 Å with its side chain pointed away from the catalytic center, and the C $_{\alpha}$ of Phe369 is displaced by 4.2 Å in towards the active site (Figure 3B,C). While Asp370 in the SNAP-25₁₉₇₋₂₀₂ and most other peptide bound structures was observed to be a key residue of the S1' subsite (8, 29–31), it is pointed away from the catalytic center in the presented structures, thereby creating a large pocket between residues Phe194 and Pro69 that accommodates the aromatic arm substituents. Interestingly, Phe369 from the PT-3 structure (but not from the PT-1 or PT-2 structures) is positioned to fill the void from the displaced Asp370 creating a hydrophobic "floor" for the S1' subsite. Similar reconfigurations in the S1' subsite were observed with the hydrophobic chlorocinnamic hydroxamate inhibitors (PDB codes 2IMA and 2ILP; (28)). However, in the PT-1 and PT-2 structures, it is residue Asn368 which replaces Asp370 to create one wall of the S1' subsite; this feature is unique to these inhibitor complexes and has not previously been reported.

Conformational Changes to the 60/70 Loop Upon Ligand Binding

Structure-based alignment of the structures in this study indicated that the largest conformational changes are primarily situated around the opening of the active site pocket (Figure 3). The 60/70 loops (residues 60–79) show the most consistent change in conformation resulting from hydrophobic interactions between Pro69 of BoNT/A LC and aromatic moieties on the S3' arm of the compounds. The Pro69 C $_{\alpha}$ positions of the inhibitor complexed BoNT/A LC structures are pulled between 2.85 to 3.1 Å into the active site when compared to uncomplexed protease (Figure 3D). These structures represent the most compact conformation of the 60/70 loop yet reported; the difference in the distance from the active site zinc atom to the Pro69 C $_{\alpha}$ atom ranges from a minimum of 2.6 Å from the nearest reported compact structure (*cf.* PT-2 to 1XTG, Table 3) to as much as a 7.1 Å difference to the most 'open' structure reported (*cf.* PT-3 to 2IMB, Table 3). The 60/70 loop conformations of the zinc- and inhibitor-bound structures presented in this study are very different from the SNAP-25₁₉₇₋₂₀₂ peptide-bound structure (PDB code 3DDA), most likely due to the presence of the bulkier peptidyl ligand in the active site (Supplemental Figure S3). In order to accommodate the P4' and P5' residues of SNAP-25, this loop is pushed away from the catalytic center resulting in Pro69 C $_{\alpha}$ displacements on the order of 6–7 Å (Table 3). Pro69 from the BoNT/A LC structure bound with the longer SNAP-25₁₄₆₋₂₀₄ peptide (PDB code 1XTG) is in a conformation similar to our P2₁ zinc-bound BoNT/A LC structure with a C $_{\alpha}$ difference of ~1.2 Å (7), although this is possibly due to the presence of active site mutations in the SNAP-25₁₄₆₋₂₀₄ bound BoNT/A LC structure which preclude native substrate-active site interactions.

Structural Comparisons with Previously Reported Hydroxamate Inhibitors

Small (~190–230 Da) "rod-like" zinc chelating hydroxamate inhibitors have previously been characterized by X-ray crystallography (28). These inhibitors are hydroxamic acid derivatives bearing either a single L-arginine arm or a 2,4-dichlorocinnamate aromatic arm that occupies the S1' subsite similar to the S1' arm of the inhibitors described in this study (Figure 4). We observe induced fit changes of the active site pocket residues that

accommodate the inhibitors that are similar to those seen in the Silvaggi study in one case (PT-3); in the other two structures there are key differences in the role of Asn368 in the formation of the S1' subsite. The conformation of Phe194 from the L-arginine hydroxamate structure (PDB code 2IMB) is similar to that found in the PT-1 structure, whereas Phe194 from the chlorocinnamic hydroxamate structures (PDB codes 2IMA and 2ILP) is similar to the PT-2 and -3 structures (Figure 4). In the PT-3 structure, Phe369 is "pulled" up into the active site to form a portion of the S1' subsite in a manner similar to the chlorocinnamic hydroxamate structures (see Figure 4A); this is in striking contrast to the structures of PT-1 and -2, in which Phe369 is rotated away from the subsite and makes no contact with the inhibitors, while Asn368 instead completes the S1' subsite. The most significant difference between the rod-like structure 2IMA and the structures reported here is in the proximity of the Pro69 residue which lies at the tip of the 60/70 loop; in the rod-like structure, Pro69 does not make inhibitor contact, whereas in each of the three structures reported here Pro69 is drawn >2.5 Å closer to the zinc, creating a portion of the hydrophobic surface of the subsite binding the S3' arm of the inhibitor, and also providing a major interaction with the S1' arm. Table 3 gives a comparison of key residue positions in these subsites.

The S2' and S3' subsites from the SNAP-25₁₉₇₋₂₀₂ BoNT/A LC structure are primarily formed by residues Arg363 and Tyr366 (8). Superposition with the SNAP-25₁₉₇₋₂₀₂ peptide-bound structure shows that the S3' arm of the compounds in this study roughly coincides with the P2' and P3' residues (Supplemental Figure S3). However, the S3' arms of our inhibitors are stacked against the 60/70 loop instead of Arg363 and Tyr366. In the structure of PT-2, the methyl ether substituent lies in a subsite which is adjacent to the zinc on the prime side of the active site, but which bears little relation to the S1' subsite occupied by Arg198 in the SNAP-25₁₉₇₋₂₀₂ peptide-bound structure. In the structures reported here, as in the other hydrophobic ligand structures (28), the displacement of Asp370 creates new prime side subsites which are significantly altered from the S1' binding site of the native substrate.

DISCUSSION

Given the exquisite potency of BoNT/A and the resulting difficulty of inhibiting the activity of the LC sufficiently to provide therapeutic benefit, the discovery and optimization of LC inhibitors as therapeutics is extremely challenging. A more complete understanding of the interactions between inhibitors and key residues in the active site is required for the design and optimization of highly potent inhibitors required to meet this challenge. Appreciating the nature and range of flexible rearrangements possible within the binding site region is essential for the design optimization of therapeutic inhibitors.

The three branched scaffold, hydroxamate-based inhibitors of BoNT/A LC are reported here for the first time. The accompanying cocrystal structures show significant commonality despite significant structural differences in the bridging scaffolds of the inhibitors. As expected, the hydroxamate moieties were observed in a classical binding orientation to the active site zinc, including displacement of a putative activated water molecule by the hydroxamate hydroxyl oxygen atom. The S1' arms of the branched inhibitors coincide with the P1' residue of SNAP-25 by binding at the S1' subsite, and the S3' arms roughly coincide with the P2' and P3' subsites through interaction with a conformationally altered 60/70 loop thereby creating a previously undescribed S2' and S3' subsite.

Significance of the 360/370 and 60/70 Loop Flexibility

In silico studies previously predicted that surface loops bordering the active site cleft would undergo conformational changes upon binding inhibitors, the largest of which involved the 60/70 loop (32). Active site flexibility, as previously noted (28), indeed plays a role in

accommodating variation in the positioning of inhibitor moieties occupying the primary binding subsites that roughly correlate to S1', S2' and S3'. While a 'switch' of Asp370 in the S1' subsite with Phe369 has been previously reported in the binding of hydrophobic small molecule inhibitors (28), the role of Asn368 in forming an S1' subsite, as in our structures for PT-1 and PT-2, has not been previously described. The structures presented here represent a 'compact' position of the 60/70 loop in which the C α of Pro69 is 2.6 – 7.1 Å closer to the active site zinc than in any previously reported BoNT/A LC crystal structures (including small molecule-bound, peptide-bound, unliganded, and holotoxin structures). This orientation of the 60/70 loop creates a compact binding site which tightly surrounds the S1' *p*-chloro- or *p*-fluorobenzyl arm of each inhibitor in the S1' subsite. The hydrophobic contacts between Pro69 and the aromatic substituent on the S3' arm of each inhibitor are likely to provide the energetic impetus for the observed movement of the 60/70 loop. Differences in the position of the 60/70 loop as well as a key hydrophobic S1' subsite residue Phe369 are likely to play a large role in the binding of disparate inhibitor classes (e.g., the rod-like hydroxamate reported in structure 2IMA). It is interesting to note that analogs of PT compounds 1–3 which lack a substituent in this arm display no inhibition of BoNT/A LC (unpublished observations). Finally, the 60/70 loop is observed to pack closely against the C-terminal region 415–424 in the holotoxin structure (PDB code 3BTA; Supplemental Figure S4). It has been noted that K_i values for small molecule inhibitors can vary significantly when tested *in vitro* against BoNT/A LC constructs with different C-terminal truncations (30); the importance of the 60/70 loop position and its interaction with the C-terminal LC region is likely to be at least partially responsible for this observation.

We suspect that binding site flexibility due to mobility of the 60/70 loop and the interplay of residue 'switching' in the 360/370 loop (Asp370/Phe369/Asn368) may be responsible for the difficulties observed by many groups in using Structure-Activity Relationship (SAR) data to guide the synthesis of more potent analogs. The structural insights gained from the analysis of these cocrystal structures is currently being used in concert with SAR data to improve the design of the next generations of BoNT/A LC inhibitors, which will be reported in due course. While the development of small molecule BoNT inhibitors as intraneuronal therapeutics remains a crucial unmet need, progress towards defining the flexible active site pharmacophore through structure-based drug design is bringing us closer to that goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

BoNT	botulinum neurotoxin
BoNT/A	botulinum neurotoxin serotype A
BoNT/A LC	botulinum neurotoxin serotype A light chain residues 1–424
SNARE	Soluble <i>N</i> -ethylmaleimide sensitive factor attachment protein receptor
SNAP-25	synaptosome-associated protein of 25,000 daltons
SNAP-25_{141–204}	SNAP-25 peptide residues 141–204

SNAP-25_{197–202}	SNAP-25peptide residues 197–202
RMSD	Root mean square deviation
60/70 loop	BoNT/A LC residues 61–79
PT-1	PanThera compound 1
PT-2	PanThera compound 2
PT-3	PanThera compound 3

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Figure 1. Overview of BoNT/A, LC, and the Chemical Structures of the Inhibitors Used in this Study

(A) BoNT/A holotoxin structure from PDB code 3BTA (4) with the LC colored gray and the HC's translocation and cell binding domain colored green and blue, respectively. (B) BoNT/A LC domain colored gray with a surface representation of the active site pocket colored blue. PT-2 (yellow sticks) is complexed with the LC deep within the active site pocket. (C) Each inhibitor contains a unique scaffold colored scarlet that bridges a zinc chelating hydroxamate moiety located on the left of each chemical structure and halogenated benzene containing arms (S1' and S3' arms).

Figure 2. Structures and Schematics of BoNT/A LC Bound by Hydroxamate Inhibitors
 1500K SA-OMIT 2|Fo|-|Fc| electron density maps contoured at 1.5 σ around the hydroxamate inhibitors (carve = 1.7 Å) with zinc ion depicted as a magenta sphere, alongside schematic representations of the enzyme-inhibitor complexes of (A) (B) PT-1, (C) (D) PT-2, and (E) (F) PT-3. The schematic shows potential hydrogen bonds represented as dashed lines and hydrophobic π - π stacking interactions as wavy lines.

Figure 3. Induced Conformational Changes Around the Active Site of BoNT/A LC
(A) Structure-based alignment of unliganded BoNT/A LC (chain A) colored gray and LC complexed with PT-1 colored green, PT-2 colored blue, and PT-3 colored orange. **(B)** Close up view of the active site with the inhibitors omitted to highlight the structural changes observed around the binding site. **(C)** Close up of 360 loop viewed from the center of the active site. **(D)** Close up of the 60/70 loop viewed from the center of the active site.

Figure 4. Structural Comparison with Previously Published Hydroxamate Inhibitors
(A) Structural alignment of BoNT/A LC structures complexed with PT-2 (colored blue) and PT-3 (colored orange) and the rod-like 2,4-Dichlorocinnamic hydroxamate inhibitor from PDB code 2IMA (colored brown; (28)). The unliganded BoNT/A LC structure (colored gray) and PT-1 complexed BoNT/A LC (colored green; inhibitor omitted for clarity) structures are also superimposed to highlight the induced fit structural changes. **(B)** Structural alignment of BoNT/A LC structures complexed with PT-1 (colored green) and the rod-like L-arginine hydroxamate inhibitor from PDB code 2IMB (colored brown; (28)). The unliganded BoNT/A LC structure (colored gray) and PT-2 and PT-3 BoNT/A LC (colored blue and orange, respectively; inhibitors omitted for clarity) structures are also superimposed to highlight the induced fit structural changes.

Table 1

Inhibition Constants for the Inhibitors against BoNT/A LC

Inhibitor	K_i (μM) $\pm$ SD
1	4.6 ± 1.8
2	6.1 ± 2.5
3	6.3 ± 0.89

Table 2

Summary of crystallographic and structure refinement data

Ligand	Unliganded	PT-1	PT-2	PT-3
PDB Code	3QIX	3QIY	3QIZ	3QJO
Space Group	$P2_1$	$P2_12_12$	$P2_12_12$	$P2_12_12$
Protomers/Atoms per ASU	2/6501	1/3241	1/3430	1/3309
Unit Cell (Å)	$a=56.0$ $b=193.7$ $c=38.8$ $\beta=96.1$	$a=59.1$ $b=190.6$ $c=42.7$	$a=59.2$ $b=190.7$ $c=42.3$	$a=59.2$ $b=188.8$ $c=42.6$
X-Ray Source	ALS 12.3.1	SSRL 11.1	SSRL 11.1	SSRL 11.1
Reflections				
Resolution Limits (Å) ^a	50–2.4 (2.49–2.4)	45–2.3 (2.38–2.3)	45–2.0 (2.07–2.0)	45–2.3 (2.38–2.3)
Total Collected	333387	713428	619655	860557
Unique	31317	20556	30442	20915
Redundancy	3.8 (3.7)	13.4 (10.5)	6.8 (4.6)	13.74 (11.9)
$\langle I \rangle / \langle \sigma(I) \rangle$	13.9 (2.6)	10.8 (4.7)	16.7 (2.5)	29.3 (16.9)
Completeness (%)	100 (99.9)	91.6 (80.7)	90.8 (79.2)	94.6 (95.4)
R _{merge} (%)	9.3 (47.8)	9.6 (32.8) ^b	8.8 (48.8)	5.8 (28.7)
Refinement				
Resolution Range (Å)	50–2.4	45–2.3	45–2.3	45–2.3
R-work (%)	18.9	22.8	18.8	20.0
R-free (%)	22.9	26.4	22.3	22.9
RMSD bond length (Å)	0.012	0.009	0.01	0.01
RMSD bond angle (°)	1.4	1.08	1.27	1.09
Mean B-factor (Å ²)				
Protein atoms	44.5	88.0	49.6	69.2
Inhibitor/Zinc	NA/86.4	91.0/57.5	48.3/31.2	71.9/48.7
Water	38.0	68.5	45.7	57.0

^aData in parentheses are for the highest resolution shell.^bThe PT-1 dataset is merged from data collected on two crystals.

Table 3

Conformational Changes within the BoNT/A LC active site pocket

Structures	Pro69 distance ^a from Zn ⁺²	Phe194 rotamer ^b	Asn368	Phe369	Asp370
LC + PT-1 (3QIY)	12.5 Å	m-85	In	Out	Out
LC + PT-2 (3QIZ)	12.6 Å	p90	In	Out	Out
LC + PT-3 (3QI0)	11.8 Å	p90	Out	In	Out
LC ^c + Zinc (3QIX)	14.6 Å	p90	In	Out	Out
LC ^c + Zinc (2ISG)	17.3 Å	m-85	In	Out	Out
LC ^c + Zinc + His-tag ^c (1XTF)	18.7 Å	m-85	Out	Out	In
LC + Zinc (2IMC)	18.1 Å	m-85	Out	Out	In
LC ^c + Zinc ^c (2ISE)	18.1 Å	m-85	Out	Out	In
LC ^c + Zinc ^c (2ISH)	18.6 Å	m-85	Out	Out	In
LC ^c + Zinc ^c (2W2D)	14.6 Å	m-85	Out	Out	In
LC Apo (3BOK)	N/A	m-85	Out	Out	In
LC + Zinc (3BON)	17.6 Å	m-30/m-85	Out	Out	In
LC + Zinc (3BWI)	18.3 Å	m-85	Out	Out	In
LC + SNAP25 ₁₄₁₋₂₀₄ (1XTG)	15.2 Å	m-85	Out	Out	In
LC + SNAP25 ₁₉₇₋₂₀₂ (3DDA)	18.2 Å	m-85	Out	Out	In
LC ^c + hydroxamate inhibitor(2IMA)	15.2 Å	p90	Out	In	Out
LC ^c + hydroxamate inhibitor (2IMB)	18.9 Å	m-85	Out	Out	In
LC + CRATKML _α (3BOO)	17.4 Å	m-85	Out	Out	In
Holotoxin _α (3BTA)	15.2 Å	m-85	Out	Out	In
Holotoxin + MAb (2NYY)	15.4 Å	m-85	Out	Out	In
Holotoxin ^c + MAb (2NZ9)	15.3 Å	m-85	Out	Out	In

^aDistances are between the C_α of Pro69 and the Zn⁺² from each respective structure.

^bRotamers defined in Lovell *et al.*, 2000 (26)

^cData for the LC + zinc structure refers to monomer A.