# Identification of a botulinum neurotoxin A protease inhibitor displaying efficacy in a cellular model

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**Herein we describe a small-molecule, non-peptidic, inhibitor for botulinum neurotoxin A protease, that displays for the first time efficacy in a cell-based assay.**

The botulinum neurotoxins (BoNTs) comprise a family of seven immunologically distinct proteins synthesized primarily by strains of the anaerobic bacteria, *Clostridium botulinum*.[1] These toxins, designated A, B, C, D, E, F and G, are the most lethal substances known with an estimated lethal intravenous dose in humans to be 1–5 ng $kg^{-1}$.[2] For all clostridial neurotoxins, toxicity results from a four-step mechanism which entails the following steps: (1) binding to ectoacceptors on the surface of motor nerve endings, (2) internalization of the toxin–receptor complex into the cytoplasm, (3) translocation of the toxin *via* a pH dependent event and (4) inhibition of impulse-evoked acetylcholine secretion *via* proteolytic cleavage of one of the SNARE proteins ultimately leading to flaccid paralysis.[2] For severe exposures, death ensues from paralysis of the diaphragm and intercostal muscles unless ventilatory support is rendered.

BoNT serotype A (BoNT/A) is the most toxic serotype of BoNTs and is considered the most threatening due to a prolonged half-life *in vivo* and ease of its production.[4] Structurally, BoNT/A consists of three functional domains; catalytic, translocation, and binding; BoNT/A toxicity results from the catalytic activity of its light chain (LC), a Zn(II) endopeptidase. The catalytic domain of BoNT/A LC is a compact globule consisting of a mixture of α-helices, β-sheets and strands with a gorge-like zinc containing metalloprotease active site 15 Å deep.[3] The metalloprotease activity is responsible for BoNT/A neurotoxicity through the hydrolytic cleavage of the SNARE (soluble NSF-attachment protein receptor) protein SNAP-25 that is involved in neuronal synaptic vesicle function.

BoNTs have been classified by the Centers of Disease Control (CDC) as one of the six highest-threat agents for bioterrorism (Class A agents), because of their extreme potency, lethality, ease of production, transport and the need for prolonged medical care.[4] Currently, an investigational pentavalent toxoid is available from the CDC and a recombinant vaccine is under development.[4] However, post-exposure vaccination is useless because of the rapid onset of the toxin. A singular drug or drug cocktail for the prevention or treatment of botulism would be enticing; yet, no small molecule has advanced to phase I clinical trials. Furthermore, while there are reports of treating BoNT/A toxicity with multiple monoclonal antibodies,[6] as antitoxins, this is of limited therapeutic utility since the antibodies must be administered prior to, or shortly after, toxin exposure (<12 h).

Presently, there are only modest small-molecule, non-peptidic, protease inhibitors for BoNT/A with $IC_{50}$ values in the range of 20 μM despite several large combinatorial libraries having been screened.[7] In addition, none of these inhibitors have demonstrated an ability to prevent SNAP-25 cleavage from BoNT/A in a cell-based assay. This latter point is important as cellular efficacy is a significant step required in any drug development program. Most of the difficulty in identifying potential BoNT/A LC protease inhibitors can be attributed to the unique nature of this protease. For example, BoNT LCs have the longest amino acid substrate requirement for any protease known.[5] This preference for an extended substrate can be attributed to the recent identification of the so called α and β exosites.[11] These exosites are peripheral substrate binding domains, which are responsible for substrate recognition unique to each BoNT LC making small molecule lead discovery even more challenging.

Recently, we established an *in vitro* high-throughput screen for the identification of inhibitors for BoNT/A LC protease.[8] Utilizing this high-throughput assay we identified a BoNT/A inhibitor. Herein we report the identification and characterization of this BoNT/A protease inhibitor that displays protection of SNAP-25 cleavage in the cell line Neuro-2a.

We initiated our studies with the idea of employing simple amino acids and their derivatives as zinc chelators for lead identification of small molecule inhibitors for BoNT/A LC.[14] During the course of these investigations, H-Cys-OH Table 1, entry 1, was found to display an $IC_{50}$ of 144 μM. Not surprisingly, the opposite enantiomer, H-D-Cys-OH, entry 2, resulted in an identical $IC_{50}$, presumably due to a lack of binding affinity of the H-Cys-OH motif with the active site zinc coordination sphere. Interestingly, and quite unexpected, an increase in potency was observed when the negative charge on the carboxylic functionality was alleviated, entry 3. Thus, in order to investigate whether the thiol moiety of entry 1 is coordinating to the zinc of BoNT/A LC, a series of thiol protected Cys, entries 4–6, were examined and found to be poorer than entry 1 or inactive. However, strikingly, when amino protected cysteines were evaluated (entry 7–9), Fmoc-Cys(trt)-OH entry 9 showed improved affinity for the BoNT/A LC. To analyze the observed increase in affinity for BoNT/A LC as seen with entry 9, several Fmoc-AA(trt)-OH amino acids were examined (entries 10–16). As anticipated, Fmoc-Ser(trt)-OH, entry 10, showed identical inhibition to entry 9, however all other amino acid derivatives analyzed lacked potency. Finally, the opposite

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**Table 1** Selection of compounds screened for inhibition of BoNT/A LC[a]

| Entry | Compound | $IC_{50}$/μM |
|---|---|---|
| 1 | H-Cys-OH | 144 ± 10 |
| 2 | H-D-Cys-OH | 142 ± 9 |
| 3 | H-Cys-OMe | 87 ± 9 |
| 4 | H-Cys(Mob)-OH | — |
| 5 | H-Cys(Bn)-OH | — |
| 6 | H-Cyc(Bn)-OEt | 151 ± 15 |
| 7 | Ac-Cys-OH | — |
| 8 | Boc-Cys-OH | — |
| 9 | Fmoc-Cys(trt)-OH | 40 ± 4 |
| 10 | Fmoc-Ser(trt)-OH | 42 ± 4 |
| 11 | Fmoc-Ser(t-Bu)-OH | — |
| 12 | Fmoc-Thr(trt)-OH | 84 ± 6 |
| 13 | Fmoc-Asn(trt)-OH | 82 ± 7 |
| 14 | Fmoc-Gln(trt)-OH | 98 ± 6 |
| 15 | Fmoc-His(trt)-OH | 91 ± 6 |
| 16 | Fmoc-Lys(trt)-OH | 153 ± 15 |
| 17 | Fmoc-D-Cys(trt)-OH | 15 ± 3 |

[a] Assays were conducted with various concentrations of inhibitor at 22.5 °C, pH 7.4 in 40 mM HEPES 0.01% (W/V) Tween® 20, 30 μM SNAPtide substrate and 300 nM enzyme according to published procedures.[8]

enantiomer of entry 9, Fmoc-D-Cys(trt)-OH, (**1**), entry 17, was evaluated and found to be remarkably more potent, suggesting a complimentary binding pocket with BoNT/A LC.

To further kinetically characterize **1**, we evaluated it in an assay system with the native SNAP-25 (141–206) substrate using HPLC for analysis. It is important to note that this region of SNAP-25 (141–206) has no structural modification, thus the structural integrity of the molecule has not been compromised. The use of such a large substrate is mandated as we have detailed, (*vide supra*), BoNT/A LC requires a larger region of substrate for optimal cleavage then typical proteases. Using this substrate, a $K_i$ of 18 ± 2 μM was found, and **1** also displayed a competitive mode of inhibition (data not shown).

In an effort to further understand the binding mode of inhibitor **1** a modeling study was performed. The crystal structure (PDB 1XTG) of BoNT/A LC with the substrate SNAP-25 (141–206) bound was used for docking studies with the program AutoDock 3.0.5.[12] For the initial protein model, all crystallographic waters, the SNAP-25 substrate, and the active site zinc were removed from the structure, and the resulting structure was used to generate energy maps with AutoGrid 3.0. The energy grid included both the active site region and the complete surface of BoNT/A LC so as to not bias the dockings into the active site. Gratifyingly, a predicted $K_i$ found in the docking study of 10 μM is in very close agreement with the experimentally observed $K_i$ of 18 μM. As gleaned from Fig. 1 a significant amount of binding energy is obtained from the Fmoc group being buried in hydrophobic pocket. Furthermore, the carboxylic acid group is in close proximity to several positively charged residues further enhancing the potency. Importantly, when **1** was converted to the hydroxamic acid[9] *via* treatment of **1** with diazomethane followed by exposure with hydroxylamine, no inhibition was observed (data not shown) thus further validating the docking model, Fig. 1, which predicts the carboxylic acid interacting with non-active site residues.

Encouraged by these findings, **1** was examined in a cell-based assay to protect against BoNT/A intoxication, using the cell line Neuro-2a.[13] While we note that PC12 cells have served as a model system for neurobiological and neurochemical studies, they are derived from adrenal pheochromocytoma cells and cannot fully mimic the *in vivo* condition with respect to BoNT sensitivity. The cell line Neuro-2a has been found to be inherently more sensitive to BoNT/A toxicity.[13] The intracellular target of BoNT/A protease within the neuroexocytosis apparatus is the soluble NSF-attachment protein receptor (SNARE) protein, SNAP-25 (synaptosomal-associated protein of 25 kDa). The BoNT/A protease cleaves the C-terminal nine amino acid residues of SNAP-25, thereby producing an approximately 24 kDa degradation protein. Analytical techniques have been developed that directly assess

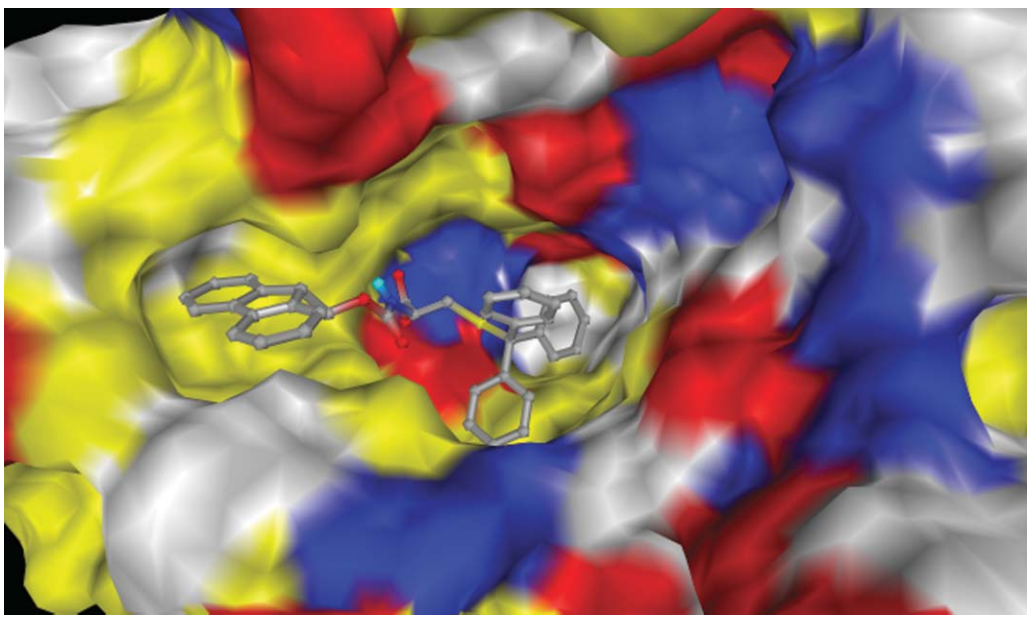

**Fig. 1** Ligand (**1**) docked into the active site of BoNT/A LC. The blue color on the molecular surface map represents negative charge, red positive charge, and yellow hydrophobicity.

 

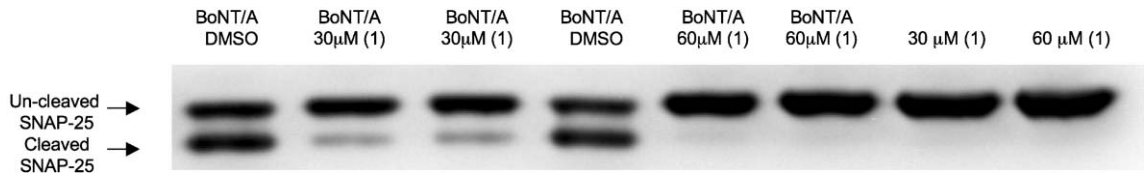


**Fig. 2** Neuro-2a (cholinergic murine neuroblastoma) cells were grown in minimum essential medium (Eagle) with Earle's salt containing 2 mM L-glutamine, 1.5 g $L^{-1}$ sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum at 37 °C in an atmosphere of 5% $CO_2$ and 95% air. Initially, the cells were grown in a 75 $cm^2$ tissue culture flask to 70–80% confluence. The cells were then used to seed a 24 well tissue culture flask at $\sim 0.5 \times 10^5$ cells per well and grown for $\sim$48 h. The media was removed, replaced with serum-free media and the cells were grown for an additional 24 h. Finally, the media was removed, replaced with serum-free media containing 2.0 μg of BoNT/A and/or **1**, and incubated for 48 h. The cells were harvested by eliminating the media, adding 75–90 μl of NuPAGE LDS sample buffer (diluted 1 : 4 with $H_2O$) followed by boiling for 10 min. The samples were analyzed by western blot using the primary antibody, SNAP-25 mouse monoclonal $IgG_1$, followed by the secondary antibody, goat antimouse HRP-conjugate. Samples were visualized using SuperSignal West Dura Chemiluminescent Substrate (Pierce) with a FluorChem 8900 (Alpha Innotech).

SNAP-25 cleavage in cell lysates using immunoelectrophoresis. Using this method, we analyzed the amount of intact *versus* cleaved SNAP-25 allowing correlation to BoNT/A activity within the cell.

As seen in Fig. 2, **1** almost completely protects the Neuro-2a cells' SNAP-25 from cleavage by BoNT/A at a concentration of 30 μM, and at 60 μM no observable cleavage can be detected. Remarkably, these concentrations are very close to the inhibition constant displayed by **1** in the protease assay. Previously, other groups have shown with BoNT/B that inhibition of pure protease *versus* cell culture required an increase of several orders of magnitude in concentration of the inhibitor for partial protection of SNARE proteins.[10] However, in our system, the concentration of protection closely mirrors the observed $K_i$.

In total, the discovery of a BoNT/A LC inhibitor, **1**, was uncovered while screening for zinc chelating amino acid lead compounds. The lead compound **1**, was evaluated in a docking study and found to be in very good agreement with our observed kinetic characterization studies. We note our docking studies have unmasked a previously unrecognized hydrophobic pocket located in the active site, which future inhibitors could be based upon. Inhibitor **1** showed protection of SNAP-25 cleavage from BoNT/A in a cell-based assay suggesting inhibition of the protease is a viable therapeutic option for reversal of botulism. Moreover, the concentration at which protection of SNAP-25 cleavage was observed closely parallels that of the observed inhibition constant, suggesting **1** has good cell permeability and intracellular access to the BoNT/A LC. Finally, this report is the first example of a BoNT/A protease inhibitor to demonstrate protection of SNAP-25 cleavage in a cell model. Further structure–activity relationship studies of **1** are currently under way and will be reported in due course.

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