EVALUATION OF A SMALL PEPTIDE INHIBITOR AND A CONTROL PEPTIDE FOR BOTULINUM NEUROTOXIN, TYPE A

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ABSTRACT

Botulinum neurotoxins are among the most toxic substances known to man. Four of the seven immunologically distinct serotypes, A, B, E, and F, cause botulism in humans. A zinc endoprotease on the 50 kDa light chain of the 150 kDa holotoxin, cleaves a single target protein essential for synaptic vesicle membrane fusion. This inhibits neurotransmitter release and leads to muscular paralysis. Measurement of this proteolytic activity provides a potentially sensitive and direct means for detecting these potent toxins. Also, the neurotoxin light chains present an ideal target for the development of potential therapeutic inhibitors. The classic approach for monitoring enzymatic activity utilizes short peptides containing a chromophore or fluorophore moiety at the site of cleavage. However, for all the botulinum serotypes, efficient cleavage requires larger peptide substrates; hydrolysis occurs only for peptides that span both sides of the cleavage site. An alternative approach is to use fluorescence resonance energy transfer (FRET) peptides. These fluorogenic peptides contain a fluorescent group at one end and a suitable chromogenic acceptor group at the other, allowing for the inclusion of amino acids on either side of the enzymatically cleaved bond. Fluorescence is quenched initially by intramolecular energy transfer between the donor/acceptor pair. Upon cleavage, the fluorescence is recovered.

A FRET peptide substrate for botulinum toxin type A (BoNT/A), SNAPtide®, has been designed based on the native synaptosomal substrate SNAP-25. Crystal structures generated with the light chain of BoNT/A and SNAPtide® show that part of the cleaved FRET substrate remains bound in the active site after hydrolysis. Based on these results, two potential peptide inhibitors have been synthesized. Data comparing the IC_{50} and K_I values for both inhibitors are presented. The peptide inhibitors can be used as positive controls in inhibitor screening assays and as lead compounds for potential BoNT/A inhibitors.

The SNAPtide® sequence was designed as an efficiently cleaved substrate for BoNT/A which contains a minimum of sites for non-specific hydrolysis. A control peptide which contains all potential non-specific cleavage sites but is not cleaved by BoNT/A has also been evaluated. Data comparing the cleavage of both the SNAPtide® substrate and the control peptide demonstrate that only minimal cleavage of the control occurs after extended exposure to relatively high amounts of BoNT/A light chain. This control peptide can be used in complex matrices to indicate the presence of contamination by non-specific enzymes.

INTRODUCTION

Evaluation of BoNT/A peptide inhibitors

The zinc dependent N-terminal light chain is the catalytic subunit which, depending on the serotype of the neurotoxin, selectively cleaves one of the SNARE membrane fusion proteins. The type A neurotoxin (BoNT/A) cleaves the 25 kDa synaptosomal protein, SNAP-25, exclusively between residues Gln^{197}-Arg^{198}. The primary sequence of the C-terminal end is given below. The minimum effective BoNT/A substrate is 13 amino acids consisting of residues 190-202 of SNAP-25 (Schmidt JJ and Bostian KA, J. Protein Chem. 1997, 16:16-26). The blue arrow indicates the BoNT/A cleavage site.

---L^{165}-D-M-G-N-E^{170}-I-D-T-Q-N^{175}-R-Q-I-D-R^{180}-I-M-E-K-A^{185}
-D-S-N-K-T^{190}-R-I-D-E-A^{195}-N-Q-R-A-T^{200}-K-M-L-G-S^{205}-G

The SNAPtide® used as the substrate in these studies is based on the native 13-amino acid sequence shown above and contains either the FRET pair, ortho amino benzoic acid (o-Abz) or fluorescein-thiocarbamoyl (FITC), on the N-terminal and a 2,4 dinitrophenyl (Dnp) or 4[[4'(dimethyl-amino)phenyl]azo]benzoic acid DABCYL on a lysine close to the C-terminal amino acid. SNAPtide® Product #520 contains the oAbZ/Dnp FRET pair while SNAPtide® Product #521 contains the FITC/DABCYL FRET pair.

A crystal structure was generated for the neurotoxin type A light chain (BoNT/A LC):SNAPtide® (oAbz/Dnp) complex (**Figure 1**). The structure shows that a part of the peptide remains bound in the active site. Specifically, the N-terminal Arg is bound in/near the P1' site and the ensuing Ala-Thr-Lys-Norleucine are well-defined. The SNAPtide® (oAbz/Dnp) peptide is definitely cleaved and Arg is the N-terminal of the fragment in the structure. Also in spite of the fact that the peptide is cleaved, and that the crystals were grown in the presence of 4mM $Zn(NO_3)_2$, there is no Zn(II) in the active site. It appears as though the presence of the 'product' may have disrupted the Zn(II)-binding site.

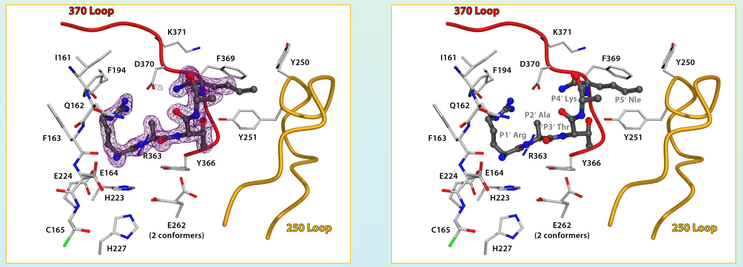

Figure 1: **Crystal structure of the botulinum neurotoxin type A light chain:SNAPtide® (oAbz/Dnp) complex.** The electron density is shown for the SNAPtide® #520 fragment remaining in the catalytic site in the image on the left. The amino acids of the fragment are labeled in the image on the right. The fragment is shown in ball-and-stick representation. The Zn^{2+} expected in coordination with E262, H227, and H223 is absent in this structure. The structure was generated in the laboratory of Karen Allen by Nick Silvaggi.

Based on the results obtained for the BoNT/A LC:SNAPtide® #520 crystal complex, two short peptides were synthesized and tested as potential inhibitors of BoNT/A. The sequences are identical except that one peptide, LBL 10506, is acetylated on the N-terminal. The sequences are:

LBL # 10505: Arg-Ala-Thr-Lys(Dnp)-Norleucine-NH_2

LBL # 10506: N-acetyl-Arg-Ala-Thr-Lys(Dnp)-Norleucine-NH_2

In the present study, these two peptides were evaluated for relative BoNT/A inhibitory potency. The inhibitory potency of SNAPtide® #520 was also evaluated for comparison.

MATERIALS AND METHODS

SNAPtide® substrates (Products #520 and #521), the Control Peptide for SNAPtide® #520 (Product #525) and Botulinum neurotoxin type A light chain (Product #610A) are products of List Biological Laboratories, Inc.

***Sample preparation*:**
Inhibitor studies: Stock solutions of the SNAPtide® (Product #521), Product #520 and LBL 10505 and LBL 10506 were made in dimethyl sulfoxide (DMSO) at 2.5, 5.0 and 50 mM, respectively. Dilutions were made at the appropriate concentrations in 50 mM HEPES, pH 7.4 containing 0.05 % Tween-20 (ASSAY BUFFER). The BoNT/A LC is prepared as a 0.25 µM solution in the ASSAY BUFFER. Details are given in the figure legends.
***Control Peptide evaluation*:** Stock solutions of the SNAPtide® (Product #520) and the Control Peptide for SNAPtide® #520 were made at 5 mM in DMSO. The peptides were diluted to the appropriate concentrations in ASSAY BUFFER. The BoNT/A LC is prepared as a 0.25 µM solution in the ASSAY BUFFER. Details are given in the figure legends.

***Fluorimetric assays*:**
Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner FLUO-TRAC black flat-bottomed plates (E&K Scientific, Campbell, CA). The time-dependent increase in fluorescence intensity was monitored at 37ºC. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the o-Abz-substrates, Products #520 and #525, and 490 nm and 523 nm using a cutoff filter of 495 nm for the FITC-substrate, Product #521.
Inhibitor studies: For assays to determine the IC_{50} and K_I values for LBL 10505,10506, and SNAPtide® #520, plates containing 10 nM BoNT/A LC and a series of dilutions of the given inhibitor were incubated at 37ºC for 30 minutes prior to addition of 10 µM SNAPtide® (Product #521) to initiate the reaction.
Control Peptide evaluation: For the assays evaluating the Control Peptide, plates containing serial dilutions of BoNT/A LC were equilibrated for 15 min at 37ºC prior to addition of 10 or 5 of µM SNAPtide® (Product #520) or the Control Peptide (Prod #525) substrate.

***Determination of IC_{50} and mode of inhibition*:** The IC_{50} values (inhibitor concentration giving 50% inhibition of BoNT/A LC) were obtained from plots of v_i/v_o where v_i is the initial velocity in the presence of the inhibitor and v_o is in the absence of inhibitor, versus concentration of inhibitor.

Dixon plots were used to determine whether the inhibition was competitive. The inverse of the initial reaction velocities, expressed as relative fluorescence units (RFU) per sec, for a series of inhibitor concentrations at three different substrate concentrations, were plotted as a function of inhibitor concentration. Values for the dissociation constant, K_I, were determined from the negative of the x-axis value at the point of intersection of the lines obtained from the three substrate concentrations.

RESULTS

IC50 values for LBL 10505, 10506, and SNAPtide® #520

As suggested by the crystal structure of the BoNT/A LC:SNAPtide® (Product #520) complex (**Figure 1**), the LBL 10505 inhibits the hydrolysis of SNAPtide® (Product #521). The IC_{50} value is 23 µM (**Figure 2**). The inhibitor that has the acetylated N-terminal, LBL 10506, is significantly less efficient at inhibiting catalysis of the FRET substrate. The IC_{50} value is 178 µM (**Figure 2**). The IC_{50} obtained for the full 13 amino acid FRET substrate, SNAPtide® #520 is 15 µM.

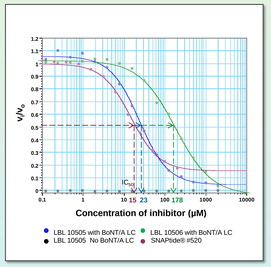

Figure 2: Dose Response curves for LBL 10505 (●) LBL 10506 (●) and SNAPtide® #520 (●). The IC_{50} values obtained for LBL 10505 is 23 µM, LBL 10506 is 178 µM and for SNAPtide® #520 the value is 15 µM.

K_I values for LBL 10505 and 10506

Dixon plots obtained for LBL 10505 and LBL 10506 using 10 nM BoNT/A LC and 5, 8, and 10 µM SNAPtide® Prod #521 are shown in **Figures 3 and 4**. The lines obtained for different substrate concentrations converge at the negative x-axis for both LBL 10505 and LBL 10506 indicating that these inhibitors behave in a competitive manner. The inhibitors compete with the substrate for binding to BoNT/A LC. The value obtained for the dissociation constant, K_I, for LBL 10505 is 19 µM while the value obtained for LBL 10506 increases by greater than 10-fold to 210 µM.

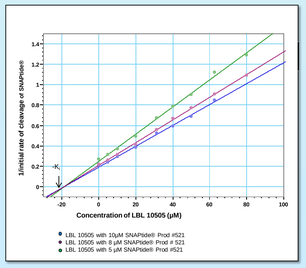

Figure 3: Dixon plots obtained for LBL 10505.

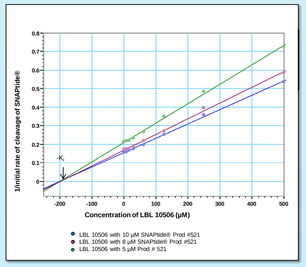

Figure 4: Dixon plots obtained for LBL 10506

Evaluation of a Control Peptide for SNAPtide® #520

The use of peptidic FRET substrates such as SNAPtide® #520 is problematic for assays performed in complicated matrices such as foodstuffs and biological fluids. A Control Peptide which is identical to SNAPtide® #520 except for two substitutions, one at the cleavage site, eliminates hydrolysis by BoNT/A LC. This Control Peptide is not a substrate for BoNT/A LC however, since it contains all of the sites for non-specific cleavage found in SNAPtide® #520, it is an ideal control peptide. As shown in **Figure 5,** the initial rate of cleavage (RFU/sec) observed for 10 and 5 µM of the Control Peptide #525 is significantly less that that observed with the same concentrations of SNAPtide® #520 even at high concentrations, 10 and 5 nM, 0.5 and 0.25 µg/ml, respectively, of BoNT/A LC. Specifically, 10 µM SNAPtide® #520 is cleaved at a rate of 4.4 RFU/sec by 10 nM BoNT/A LC while an equal amount of Control Peptide #525 is cleaved at a rate ~ 140 times less at 0.031 RFU/sec by the same amount of BoNT/A LC. The fluorescence intensities observed after 2.5 hrs incubation with BoNT/A LC are shown in **Figure 6**. Only a slight increase in RFU above the zero BoNT/A value is observed for the Control Peptide #525 at the 5 and 10 nM BoNT/A points.

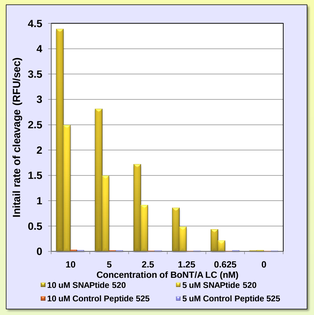

Figure 5: Initial rate of cleavage (RFU/sec) for SNAPtide® #520 and Control Peptide #525 as a function of BoNT/A LC concentration. Two concentrations of SNAPtide® #520 and Control Peptide #525,10 and 5 µM, were incubated with a series of concentrations of BoNT/A LC at 37°C (see legend).

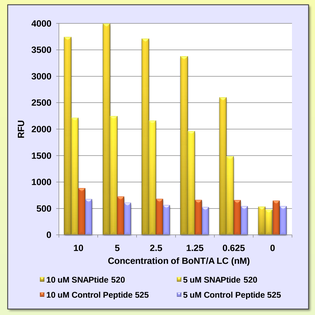

Figure 6: Fluorescence intensities (RFU) observed after 2.5 hrs. incubation with BoNT/A LC as a function of BoNT/A LC concentration. Two concentrations of SNAPtide® #520 and Control Peptide #525,10 and 5 µM, were incubated with a series of concentrations of BoNT/A LC at 37°C for 2.5 hrs. (see legend).

CONCLUSIONS

Evaluation of BoNT/A peptide inhibitors

A. IC_{50} measurements

Two five residue peptides, LBL 10505 and 10506, corresponding to the cleaved fragment of the FRET BoNT/A substrate, SNAPtide®, were evaluated by IC_{50} measurements. One of the fragments, LBL 10506, is acetylated on the N-terminal Arg. The data indicates that the inhibitory capacity of these peptides is significantly compromised by N-acetylation. The IC_{50} values are 23 µM for LBL 10505, with the free amino group at the N-terminus, versus 178 µM for LBL 10506. This is consistent with what has been observed previously (Zuniga et al., Structure, 2008, 16:1588-1597) for a series of 7 residue peptide inhibitors based on the native SNAP-25 substrate with various modifications. N-acetylation decreased inhibition compared to the derivatives that had a free amino group at the N-terminus. In order to determine if generation of the fragment LBL 10505 from SNAPtide® #520 at the cleavage site would influence the inhibition, the IC_{50} for the FRET substrate was determined independently. The IC_{50} value for the FRET substrate, 15 µM, is in the range observed for the cleaved fragment LBL 10505 alone.

B. Inhibitor dissociation constants, K_I and mode of inhibition.

Dissociation constants measured for LBL 10505 and 10506, 19µM and 210 µM respectively, are consistent with the IC_{50} values (see above). The N-acetyl group at the N-terminus of LBL 10506 increases the K_I 10-fold similar to the increase in the IC_{50}. The Dixon plots indicate that both LBL 10505 and 10506 compete with the substrate for binding to the BoNT/A LC.

C. Absence of Zn(II) in the catalytic site.

A number of crystal structures have been generated with small peptide inhibitors of BoNT/A LC (Zuniga et al., Structure, 2008, 16:1588-1597; Silvaggi et al., Biochemistry, 2008, 47:5736-5745; Kumaran et al., J. Biol. Chem. 2008, 18883-18891; Kumaran et al., Plos Pathogens, 2008 4:e1000165). The BoNT/A LC:small peptide inhibitor complexes include specific contacts with Zn(II) in the catalytic site. For the crystal structures presented here (**Figure 1**), the Zn(II) is absent. This suggests that SNAPtide® #520 may act as a suicide inhibitor. An IC_{50} value for this SNAPtide® #520 was obtained to examine this hypothesis. The IC_{50} values for SNAPtide® #520 and LBL 10505 are similar so catalysis of the substrate to generate the LBL 10505 sequence is not necessary. The absence of the Zn(II) in the crystal structure is unexplained.

Evaluation of a Control Peptide for SNAPtide® #520

A control peptide designed to contain the sequence of SNAPtide® #520 with minimal modifications, Product #525, was evaluated. The results confirm that the cleavage of Product #525 by BoNT/A is severely compromised. All potential non-specific cleavage sites remain available in the Product #525 sequence. This Control Peptide can be used to screen background cleavage of SNAPtide® #520 that can occur in complex matrices limiting the detection of low amounts of BoNT/A.