A high-affinity competitive inhibitor of type A botulinum neurotoxin protease activity

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Abstract **The peptide *N*-acetyl-CRATKML-amide is an effective inhibitor of type A botulinum neurotoxin (BoNT A) protease activity [Schmidt et al., FEBS Lett. 435 (1998) 61–64]. To improve inhibitor binding, the peptide was modified by replacing cysteine with other sulfhydryl-containing compounds. Ten peptides were synthesized. One peptide adapted the structure of captopril to the binding requirements of BoNT A, but it was a weak inhibitor, suggesting that angiotensin-converting enzyme is not a good model for BoNT A inhibitor development. However, replacing cysteine with 2-mercapto-3-phenylpropionyl yielded a peptide with K_i of 330 nM, the best inhibitor of BoNT A protease activity reported to date. Additional modifications of the inhibitor revealed structural elements important for binding and supported our earlier findings that, with the exception of P_1' arginine, subsites on BoNT A are not highly specific for particular amino acid side chains.**

Key words: Botulinum neurotoxin; Protease inhibitor

1. Introduction

Botulinum neurotoxins (BoNTs) are produced by various strains of the anaerobic spore-forming bacilli *Clostridium botulinum*, *C. butyricum* and *C. baratii* [1–3]. There are seven serotypes, A–G [4]. Each neurotoxin consists of a heavy chain (M_r ~100 000) and a light chain (M_r ~50 000), covalently linked by a single disulfide bond. The heavy chain includes domains for binding to peripheral motor neurons and toxin internalization, while the light chain is a zinc metalloprotease, specific for proteins involved in acetylcholine release. BoNTs A, C, and E cleave synaptosomal-associated protein of M_r 25 000 (SNAP-25), while synaptobrevin is the target of B, D, F, and G. In addition to SNAP-25, BoNT C also cleaves syntaxin. Each neurotoxin hydrolyzes only a single bond in its substrate, but this is sufficient to prevent exocytosis of neurotransmitter [5,6].

Botulinum neurotoxins are among the most potent toxins known [7]. Nonetheless, they have proven to be useful tools in neurophysiology studies [4,8,9] and as drugs to treat certain muscle dysfunctions and other diseases in humans [7,10–13]. Therefore, considerable interest in BoNTs has developed with regard to catalytic properties [14–16], three-dimensional structures [17–20], BoNT internalization and intracellular processing [5], and development of specific inhibitors to reverse or modulate BoNT-induced paralysis [21–23].

In earlier work, we developed a practical high-performance liquid chromatography (HPLC)-based assay for BoNT A protease activity, and unequivocally demonstrated that the 'SNARE motifs' [24,25] in SNAP-25 are not required for BoNT A substrate recognition [15]. We then employed the assay to characterize the catalytic properties of BoNT A [16], and used this information to obtain the first specific competitive inhibitors of BoNT A protease activity [22]. In this report, we describe development of the first BoNT A inhibitor with K_i in the nanomolar range, present findings on the contributions of inhibitor side chains to binding affinity, and discuss implications of our results with respect to further BoNT A inhibitor development.

2. Materials and methods

2.1. Peptide synthesis

The peptide synthesizer was a model 431A from Applied Biosystems (Foster City, CA, USA). We used reagents and protocols obtained from the same source. Peptides were synthesized as C-terminal amides, and purified by reverse phase HPLC with gradients of acetonitrile in 0.1% trifluoroacetic acid (TFA). Equipment for HPLC was from Waters Corporation (Milford, MA, USA). HPLC columns (Hi-Pore RP318 and RP304) were from Bio-Rad Laboratories (Hercules, CA, USA).

In the peptides synthesized for this study, the N-terminal moieties contained sulfhydryl groups and were not standard amino acids. In some cases, the appropriate S-protected compounds were commercially available from Calbiochem-Novabiochem (San Diego, CA, USA) or Bachem (King of Prussia, PA, USA). Others were custom-synthesized by Bachem. Because custom syntheses of N-terminal intermediates containing optically active carbons were not stereospecific, each subsequent peptide yielded a racemic mixture, which was then purified by HPLC. The earlier-eluting stereoisomer was designated peptide A, while the later was peptide B.

2.2. Assay of BoNT A protease activity

BoNT A was obtained from Metabiologics (Madison, WI, USA). Assays of BoNT A protease activity were performed as described previously [15,16]. Briefly, assays (30 μl) contained 20 mM HEPES, pH 7.3, 1 mg/ml bovine serum albumin, 5 mM dithiothreitol, 0.25 mM $ZnCl_2$, various concentrations of substrate (a peptide corresponding to residues 187–203 of SNAP-25), and 1 μg/ml BoNT A. The temperature was 37°C. Assays were stopped by adding TFA to 0.5%, then analyzed by reverse-phase HPLC. Concentrations of substrates and hydrolysis products were calculated from peak areas.

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Abbreviations: BoNT, the protein neurotoxin produced by *Clostridium botulinum*; HPLC, high-performance liquid chromatography; mpp, 2-mercapto-3-phenylpropionyl; SNAP-25, synaptosomal-associated protein of M_r 25 000; TFA, trifluoroacetic acid

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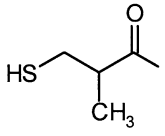
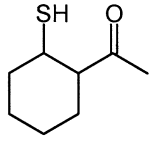
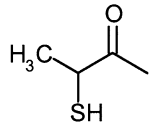
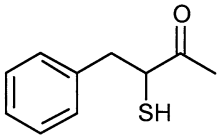
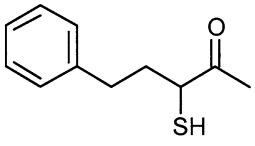
Kinetic constants were obtained from plots of initial rates with seven concentrations of substrate. Results were calculated from non-linear regression analyses by using the program Enzfitter (Biosoft, Cambridge, UK). Values are the averages of three independent determinations. In all cases, standard deviations were less than ±20%. K_i values for the inhibitors were calculated in triplicate from Dixon plots with the equation: $K_i = K_m/[(\text{slope})(V_{max})[S]]$, where [S] is the substrate concentration [26]. For the best inhibitor, K_i and the type of inhibition were also evaluated by determining substrate kinetic constants in the presence of five different inhibitor concentrations. K_i values for each of the five assays in the presence of inhibitor were calculated with the equation: $K_i = [I]/[(K_{mapp}/K_m)-1]$, where [I] is inhibitor concentration [26].

3. Results and discussion

3.1. K_i values of the inhibitors

In our earlier report [22], we presented evidence that the relatively strong binding of the BoNT A inhibitor CRATKML ($K_i = 2$ μM) was probably due to binding of BoNT A active site zinc by the N-terminal cysteine sulfhydryl group. Therefore, to obtain an inhibitor of BoNT A with lower K_i than CRATKML, we retained the sulfhydryl but modified other aspects of the N-terminal moiety. Two strategies were employed: (1) the sulfhydryl was kept on the β carbon, and the amino on the α carbon was replaced with hydrogen and with other groups; (2) the α amino was eliminated, the sulfhydryl was moved to the α carbon, and the β carbon was modified. After synthesizing each compound, it was attached through an amide bond to the N-terminus of the peptide RATKML, and the K_i value of the product was determined. Results for peptides containing representative compounds from the two groups are shown in Table 1.

Table 1
Structures of N-terminal moieties and K_i values of peptide derivatives

XRATKML(amide) where "X" is:	Stereoisomer	K_i (μM)
	1A	50
	1B	20
	2A	70
	2B	100
	3A	1
	3B	30
	4A	8
	4B	0.3
	5A + 5B	2

Peptide 1 in Table 1 has 2-methyl-3-mercaptopropionyl as the N-terminal moiety. This peptide is included in the table because it can be considered an analog of the anti-hypertension drug captopril, which is *N*-(2-methyl-3-mercaptopropionyl)-proline [27]. Captopril binds tightly ($K_i = 0.3$ nM) to its target protein, angiotensin-converting enzyme, a zinc carboxydipeptidase [27]. However, captopril was reported to be a weak inhibitor of BoNT protease activity [28,29], probably because none of the botulinum toxins can bind proline near the active site. Therefore, we anticipated that replacing the proline in captopril with RATKML would adapt the molecule to fit the binding requirements of BoNT A and yield a good inhibitor. However, neither the A nor the B isomer of peptide 1 had a lower K_i relative to our earlier inhibitor [22]. We concluded that angiotensin-converting enzyme is not a good model for development of BoNT A protease inhibitors.

The N-terminal moiety of peptide 2 retains the sulfhydryl group on the β carbon, but the α and β carbons are included in a cyclohexane ring. Introducing a cyclic structure was an attempt to decrease rotational freedom (lower entropy) and thereby enhance binding of the peptide, a common strategy for developing enzymatic inhibitors [30,31]. However, neither peptide 2A nor 2B bound strongly to BoNT A.

In contrast to the above results, transfer of the sulfhydryl to the α carbon yielded high-affinity inhibitors of BoNT A protease activity. Peptide 3A, with 2-mercaptopropionyl as the N-terminal moiety, exhibited a modest but significant improvement in K_i, compared to the cysteine peptide. This finding suggested that an α sulfhydryl was in a more favorable position for binding the active site zinc compared to a β sulfhydryl. A phenyl ring was then added to the β carbon to produce peptide 4. The later-eluting stereoisomer, 4B, had the lowest K_i value of any BoNT A inhibitor described to date, 0.3 μM.

Finally, a methylene group was added to the carbon chain of the N-terminal moiety to yield peptide 5. However, we were unable to separate the two peptide stereoisomers, probably because of increased rotational freedom caused by the lengthened carbon chain. The K_i value of the 5A+5B mixture was 2 μM (Table 1), while that for an equimolar mixture of 4A and 4B was 0.7 μM. If we assume that the two stereoisomers of peptide 5 were present in equimolar amounts (which was true for all other peptides in this study), it is unlikely that either 5A or 5B has a K_i value lower than that of 4B.

Other peptides with various N-terminal moieties were synthesized and tested, but are not shown in Table 1. They included peptides where X was: 3-mercaptopropionyl, thioglycolyl, L-homocysteinyl, L-penicillaminyl, and D-penicillaminyl. K_i values ranged from 2 μM to greater than 10 mM (data not shown).

3.2. N-terminal conformations of peptides 3 and 4

The N-terminus of peptide 3 is an analog of alanine, with sulfhydryl on the α carbon instead of an amino group. Similarly, peptide 4 N-terminus is an analog of phenylalanine. Peptides XRATKML were synthesized where X was L-ala-

Table 2
Effects of inhibitor on substrate kinetic constants

[I][a] (μM)	V_{max} (μmol/min/mg)	K_{mapp} (mM)	K_i (μM)
0	15	1.3	
0.087	14	1.6	0.37
0.16	14	2.1	0.27
0.26	15	2.4	0.30
0.33	14	2.5	0.36
0.61	16	4.3	0.27

[a][I] is peptide 4B, mpp-RATKML.

nine, D-alanine, L-phenylalanine, and D-phenylalanine. K_i values for these peptides were 6, 18, 20, and 150 μM, respectively, showing that within each pair, the peptide with the L-amino acid at the N-terminus was a more effective inhibitor than the corresponding D-amino acid peptide. HPLC retention time of L-ARATKML was earlier than the D-peptide, but the converse was true for the two stereoisomers of FRATKML. This pattern was mirrored precisely by the stereoisomers of peptides 3 and 4, i.e. the best inhibitors were the earlier-eluting 3A and the later-eluting 4B. Therefore, we concluded that the absolute conformations about the N-terminal alpha carbons in peptides 3A and 4B probably correspond to the L-forms of naturally occurring amino acids.

3.3. *Peptide 4A is a competitive inhibitor of BoNT A protease activity*

Because Dixon plots do not indicate the type of inhibition (competitive, non-competitive, or uncompetitive), we compared kinetic constants determined for the 17-residue peptide substrate alone to those obtained in the presence of five concentrations of peptide 4B. Results are shown in Table 2. Peptide 4B had no effect on V_{max}, but K_{mapp} increased with inhibitor concentration. Therefore, peptide 4B was a competitive inhibitor of BoNT A protease activity. The average K_i was 330 ± 50 nM. Preincubating BoNT A with peptide 4B for up to 30 min had no effect on the extent of inhibition, compared to that observed when BoNT A was simultaneously exposed to substrate and inhibitor. Therefore, the K_i value for peptide 4B probably represents equilibrium binding of inhibitor to enzyme (data not shown).

3.4. *Effects of inhibitor peptide length and amino acid substitutions on K_i values*

In earlier work [22], we obtained good inhibition of BoNT A protease activity by substituting cysteine for P_1 glutamine in the 17-residue substrate peptide. We then found that eliminating all the residues on the N-terminal side of the cysteine had no effect on inhibitor binding. In this study, we determined the contributions to binding of residues on the C-terminal side of the 2-mercapto-3-phenylpropionyl (mpp) group (which corresponds to the substrate P_1 residue) by synthesizing and testing truncated versions of peptide 4. K_i values for the B-isomers, which in all cases were lower than those of the A-isomers, are shown in Table 3. Both mpp-R and mpp-RA had the same K_i, 60 μM, while adding threonine lowered it by 50%. However, additions of the lysine and methionine residues had the strongest effects on K_i, with approximately seven-fold and 10-fold decreases, respectively. Including leucine and GSG, the last four residues of SNAP-25, had no effect on K_i.

In the inhibitors, residues RATKM correspond to the P_1' through P_5' residues of the substrate. To investigate further the binding contribution of each amino acid side chain, we substituted other amino acids, one substitution per peptide, at these locations in peptide 4B (Table 3). Because of their relatively strong contributions to inhibitor binding affinity, the effects of replacing P_5' methionine and P_4' lysine were of particular interest. However, substitution of alanine for methionine caused only a small increase in K_i, suggesting that the presence of a peptide bond in that location was of more consequence than the structure of the side chain. Replacing lysine with alanine caused a 10-fold increase in K_i, but this was less drastic than might have been expected, given the substitution of a small hydrophobic residue for a large positively charged amino acid and the importance of the P_4' residue for binding. Similar results were found when P_3' threonine was replaced with alanine, and P_2' alanine with the branched amino acid valine. In contrast, P_1' arginine could not be replaced by lysine (shown) or alanine or citrulline (not shown) without drastic loss of binding.

In summary, these findings support our earlier conclusion that, with the exception of P_1' arginine, BoNT A does not have absolute requirements for particular side chains with respect to binding of substrates or inhibitors [16,22]. In peptide 4B, the major contributors to binding affinity are the sulfhydryl group (presumably binding the active site zinc), the phenyl ring, the arginine side chain, and the peptide backbone of the first five amino acids. Therefore, it is unlikely that an inhibitor with significantly lower K_i would result from replacements of RATKM in peptide 4 with other amino acids, either individually or in combinations. Similarly, the improvement in binding affinity obtained by adding the phenyl ring to peptide 3 to yield peptide 4 could not have been anticipated, because the subsite on BoNT A for side chains at this location (equivalent to the P_1 residue of the substrate) was also shown to be non-specific with respect to binding [16,22].

Nonetheless, the work described herein has resulted in the most effective inhibitor of BoNT A protease activity to date: peptide 4B, with K_i of 330 nM. Because it is a competitive inhibitor, peptide 4B will prove useful in studies of BoNT A active site and interactions with substrate, through X-ray analyses of BoNT A–peptide 4B cocrystals. It will also serve as a model for further development of inhibitors with enhanced binding and improved biostability properties, from additional modifications to the N-terminal moiety and replacement of the other residues with non-amino acid com-

Table 3
Effects of inhibitor peptide length and amino acid substitutions on K_i

Inhibitor	K_i[a] (μM)
mpp-R	60
mpp-RA	60
mpp-RAT	30
mpp-RATK	4
mpp-RATKM	0.3
mpp-RATKML	0.3
mpp-RATKMLGSG	0.3
mpp-RATKAL	0.7
mpp-RATAML	3
mpp-RAAKML	0.7
mpp-RVTKML	2
mpp-KATKML	> 300

[a]K_i values are for the B-stereoisomers.

pounds. A wide range of modifications must be evaluated, and the work extended to include other BoNT serotypes. To this end, we have developed true high-throughput assays for the protease activities of BoNTs A, B, D, and F [32], and work on similar assays for serotypes C, E, and G is in progress.

References

[1] Simpson, L.L. (1986) Annu. Rev. Pharmacol. Toxicol. 26, 427–453.
[2] Hall, J.D., McCroskey, L.M., Pincomb, B.J. and Hatheway, C.L. (1985) J. Clin. Microbiol. 21, 654–655.
[3] Aureli, P., Fenicia, L., Pasolini, B., Gianfranceschi, M., McCroskey, L.M. and Hatheway, C.L. (1986) J. Infect. Dis. 154, 207–211.
[4] Schiavo, G., Matteoli, M. and Montecucco, C. (2000) Physiol. Rev. 80, 717–766.
[5] Humeau, Y., Doussau, F., Grant, N.J. and Poulain, B. (2000) Biochimie 82, 427–446.
[6] Schiavo, G., Rossetto, O., Tonello, F. and Montecucco, C. (1995) in: Clostridial Neurotoxins (Montecucco, C., Ed.), pp. 257–254, Springer-Verlag, Berlin.
[7] Kessler, K.R. and Beneke, R. (1997) NeuroToxicology 18, 761–770.
[8] Niemann, H., Blasi, J. and Jahn, R. (1994) Trends Cell Biol. 4, 179–185.
[9] Schiavo, G., Rossetto, O. and Montecucco, C. (1994) Cell Biol. 5, 221–229.
[10] Montecucco, C., Schiavo, G., Tugnoli, V. and de Grandis, D. (1996) Mol. Med. Today 2, 418–424.
[11] Tsui, J.K. (1996) Pharmacol. Ther. 72, 13–24.
[12] Heckmann, M., Ceballos-Baumann, A.O. and Plewig, G. (2001) New Engl. J. Med. 344, 488–493.
[13] Rosetto, O., Seveso, M., Caccin, P., Schiavo, G. and Montecucco, C. (2001) Toxicon 39, 27–41.
[14] Shone, C.C. and Roberts, A.K. (1994) Eur. J. Biochem. 225, 263–270.
[15] Schmidt, J.J. and Bostian, K.A. (1995) J. Protein Chem. 14, 703–708.
[16] Schmidt, J.J. and Bostian, K.A. (1997) J. Protein Chem. 16, 19–26.
[17] Umland, T.C., Wingert, L.M., Swaminathan, S., Furey, W.F., Schmidt, J.J. and Sax, M. (1997) Nature Struct. Biol. 4, 788–792.
[18] Lacy, D.B., Tepp, W., Cohen, A.C., DasGupta, B.R. and Stevens, R.C. (1998) Nature Struct. Biol. 5, 898–902.
[19] Hanson, M.A. and Stevens, R.C. (2000) Nature Struct. Biol. 7, 687–692.
[20] Swaminathan, S. and Eswaramoorthy, S. (2000) Nature Struct. Biol. 7, 693–699.
[21] Adler, M., Nicholson, J.D., Cornille, F. and Hackley, B.E. (1998) FEBS Lett. 429, 234–238.
[22] Schmidt, J.J., Stafford, R.G. and Bostian, K.A. (1998) FEBS Lett. 435, 61–64.
[23] Martin, L., Cornille, F., Turcaud, S., Meudal, H., Roques, B.P. and Fournie-Zaluski, M.C. (1999) J. Med. Chem. 42, 515–525.
[24] Rossetto, O., Schiavo, G., Montecucco, C., Poulaun, B., Deloye, F., Lozzl, L. and Shone, C. (1994) Nature 372, 415–416.
[25] Washbourne, P., Pellizari, R., Baldini, G., Wilson, M. and Montecucco, C. (1997) FEBS Lett. 418, 1–5.
[26] Segel, I.H. (1975) Enzyme Kinetics, Wiley, New York.
[27] Powers, J.C. and Harper, J.W. (1986) in: Proteinase Inhibitors (Barret, A.J. and Salvesen, G., Eds.), pp. 219–298, Elsevier, New York.
[28] Foran, P., Shone, C. and Dolly, J.O. (1994) Biochemistry 33, 15365–15374.
[29] Adler, M., Deshpande, S.S., Sheridan, R.E. and Lebeda, F.J. (1994) in: Therapy with Botulinum Toxin (Jankovic, J. and Hallet, M., Eds.), pp. 63–70, Marcel Dekker, New York.
[30] Browner, M.F., Smith, W.W. and Castelhano, A.L. (1995) Biochemistry 34, 6602–6610.
[31] Bohacek, R., De Lombaert, S., McMartin, C., Priestle, J. and Grütter, M. (1996) J. Am. Chem. Soc. 118, 8231–8249.
[32] Schmidt, J.J., Stafford, R.G. and Millard, C.B. (2001) Anal. Biochem. 296, 130–137.