Structural Basis for BABIM Inhibition of Botulinum Neurotoxin Type B Protease

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Design of peptidase inhibitors by rational modification of the substrate sequence is a powerful method for drug discovery, providing, for example, the ACE inhibitors[1] and the HIV protease inhibitors.[2] This process applies transition-state analogue inhibition principles[3] to peptide substrates to generate peptide-derived inhibitors.[4] These structures are then modified by peptidomimetic substitutions[5] to obtain pharmaceutically useful compounds. However, this strategy fails when the minimal enzyme substrate is too large to be mimicked by small molecules. Such is the case with inhibitors designed against the botulinum neurotoxin (BoNT) catalytic domains, which are the metalloproteases responsible for the toxic action associated with these neurotoxins.

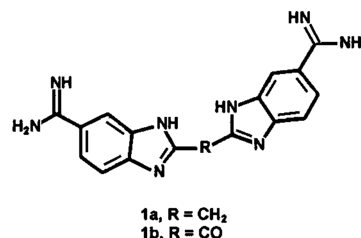
Herein, we describe the discovery of novel non-peptide inhibitors of BoNT serotype B metalloprotease (BoNT/B-LC) and show that one inhibitor binds to the enzyme in a novel orientation. Our results show that low molecular mass, non-peptide inhibitors of highly selective peptidases can be obtained by utilizing nonsubstrate binding sites.

The anaerobic bacteria *Clostridium botulinum* (serotypes A−G) produce neurotoxins whose catalytic zinc protease domain cleaves one of three SNARE proteins responsible for neurotransmitter vesicle fusion, blocking the release of neurotransmitters and producing the paralytic symptoms of the disease botulism.[6] These peptidases are the most selective yet described with minimum substrate length requirements varying from 17 to 55 amino acids, depending on the serotype. For example, BoNT/B-LC cleaves only peptides containing at least 35 amino acids.[7] The remarkable specificity associated with these zinc-dependent endopeptidases can be attributed to the requirement that the toxin binds two distinct regions of the substrate,[8] thereby activating the enzyme and enabling catalysis.[9,10] The substrate must be long enough to contain both recognition sequences separated by a critical distance that is serotype specific. These structural constraints dictate the minimum substrate length that can be effectively proteolyzed.

The rigorous substrate specificity and length requirements are reflected in the difficulty associated with identifying good inhibitors to this class of enzyme. Known inhibitors of other extensively investigated metalloproteases are 6 orders of magnitude weaker inhibitors of the botulinum and tetanus neurotoxin proteases.[11−13] For example, captopril, thiorphan, and phosphoramidon, all nanomolar inhibitors of enzymes related to thermolysin, do not inhibit BoNT/B-LC except at millimolar concentrations. Zinc chelators are moderate inhibitors but highly toxic in vivo.[14] ICD1578, an isocoumarin human leukocyte elastase inhibitor, weakly inhibits BoNT/B-LC protease.[15] Moderately effective peptide-derived inhibitors of *Clostridium* neurotoxin proteases A and tetanus neurotoxin have been reported.[16−18]

We tested bis(5-amidino-2-benzimidazolyl)methane (BABIM; **1a**) and keto-BABIM (**1b**)[19,20] for inhibition of BoNT/B-LC protease following the report that **1a** inhibited serine proteases[21] by a zinc complex formed between the bis-imidazoyl moiety in **1a** and the enzyme's active-site histidine and serine residues. Inhibition of BoNT/B-LC protease was determined by analytical HPLC using the reported procedure[22] on synthetic synaptobrevin (Sb2; residues 60−94), the minimum substrate for BoNT/B-LC. The K_i for BABIM was 1.6 ± 0.2 μM, and initial velocity data best fit a linear competitive inhibition model. Keto-BABIM was found to be a better inhibitor ($K_i = 0.8 \pm 0.4$ μM). Although BABIM weakly binds zinc (52 μM),[23] the excellent zinc chelator 1,10-phenanthroline (Log $K_{Zn-Phen} = 6.55$[24]) did not inhibit BoNT/B-LC protease at <50 μM. Thus, inhibition by these molecules is not caused by the removal of active-site zinc from BoNT/B-LC protease.

The X-ray structure of the catalytic domain of BoNT/B-LC cocrystallized with BABIM confirmed that the inhibitor binds in



1a, R = CH2
1b, R = CO

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(1) Ondetti, M. A.; Rubin, B.; Cushman, D. W. *Science* **1977**, *196*, 441−444.
(2) Wlodawer, A.; Erickson, J. W. *Annu. Rev. Biochem.* **1993**, *62*, 543−585.
(3) Pauling, L. *Chem. Eng. News* **1946**, *24*, 1375. Wolfenden, R. *Acc. Chem. Res.* **1972**, *5*, 10−18.
(4) Rich, D. H. Peptidase Inhibitors. In *Comprehensive Medicinal Chemistry. The Rational Design, Mechanistic Study and Therapeutic Application of Chemical Compounds*; Hansch, C., Sammes, P. G., Taylor, J. B., Eds.; Pergamon Press: Elmsford, NY, 1990; pp 391−441.
(5) Recent Advances in "Peptidomimetic Design". Ripka, A. S.; Rich, D. H. *Curr. Opin. Chem. Biol.* **1998**, *4*, 439−452.
(6) Montecucco, C.; Schiavo, G. *Q. Rev. Biophys.* **1995**, *28*, 423−472 and references therein.
(7) Shone, C. C.; Quinn, C. P.; Wait, R.; Hallis, B.; Fooks, S. G.; Hambleton, P. *Eur. J. Biochem.* **1993**, *217*, 965−971.
(8) Pellizzari, R.; Rossetto, O.; Lozzi, L.; Giovedi, S.; Johnson, E.; Shone, C. C.; Montecucco, C. *J. Biol. Chem.* **1996**, *271*, 20353−20358.
(9) Cornille, F.; Martin, L.; Lenoir, C.; Cussac, D.; Roques, B. P.; Fournie-Zaluski, M. *J. Biol. Chem.* **1997**, *272*, 3459−3464.
(10) Hanson, M. A.; Stevens, R. C. *Nat. Struct. Biol.* **2000**, *7*, 687−692.
(11) Foran, P.; Shone, C. C.; Dolly, O. *Biochemistry* **1994**, *33*, 15365−15374.
(12) Cornille, F.; Goudreau, N.; Ficheux, D.; Niemann, H.; Roques B. P. *Eur. J. Biochem* **1994**, *222*, 173−181.
(13) Adler, M.; Nicholson, J. D.; Starks, D. F.; Kane, C. T.; Cornille, F.; Hackley, B. E., Jr. *J. Appl. Toxicol.* **1999**, *19*, S5−S11.
(14) Adler, M.; Dinterman, R. E.; Wannemacher, R. W. *Toxicon* **1997**, *35*, 1089−1100.
(15) Adler, M.; Nicholson, J. D.; Hackley, B. E. *FEBS Lett.* **1998**, *429*, 234−238.
(16) Schmidt, J. J.; Stafford, R. G.; Bostian, K. A. *FEBS Lett.* **1998**, *435*, 61−64.
(17) Martin, L.; Cornille, F.; Coric, P.; Roques, B. P.; Fournie-Zaluski, M.-C. *J. Med. Chem.* **1998**, *41*, 3450−3460.
(18) Martin, L.; Cornille, F.; Turcaud, S.; Meudal, H.; Roques, B. P.; Fournie-Zaluski, M.-C. *J. Med. Chem.* **1999**, *42*, 515−525.
(19) Fairley, T. A.; Tidwell, R. R.; Donkor, I.; Naiman, N. A.; Ohemeng, K. A.; Lombardy, R. J.; Bentley, J. A.; Cory, M. *J. Med. Chem.* **1993**, *36*, 1746−1753.
(20) Tidwell, R. R.; Geratz, J. D.; Dubovi, E. J. *J. Med. Chem.* **1983**, *26*, 294−298.
(21) Katz, B. A.; Clark, J. M.; Finer-Moore, J. S.; Jenkins, T. E.; Johnson, C. R.; Ross, M. J.; Luong, C.; Moore, W. R.; Stroud, R. M. *Nature* **1998**, *391*, 608−612.
(22) Shone, C. C.; Quinn, C. P.; Wait; R.; Hallis, B.; Fooks, S. G.; Hambleton, P. *Eur. J. Biochem.* **1993**, *217*, 965−971.
(23) Janc, J. W.; Clark, J. M.; Warne, R. L.; Elrod, K. C.; Katz, B. A.; Moore, W. R. *Biochemistry* **2000**, *39*, 4792−4800.
(24) Mitchell, P. R.; Sigel, H. *J. Am. Chem. Soc.* **1978**, *100*, 1564−1570.

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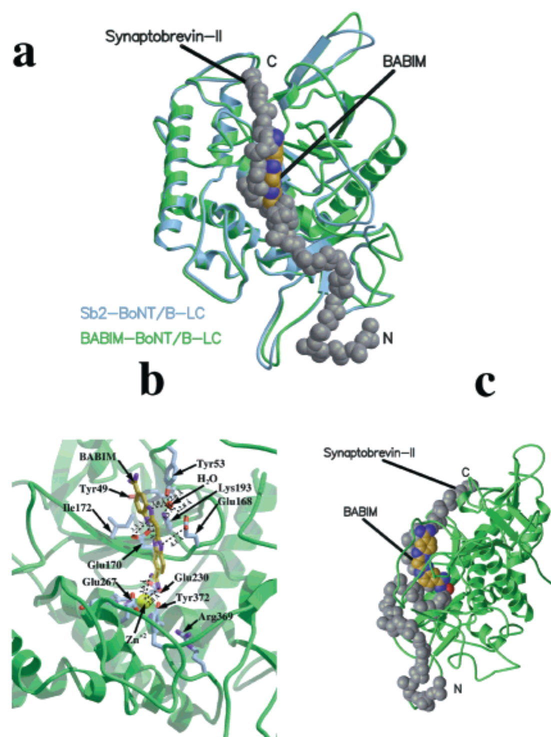

Figure 1. (a) An overlay of BABIM-BoNT/B-LC shown in green with Sb2-BoNT/B-LC shown in blue. The synaptobrevin peptide is in gray, and BABIM is colored with gold carbon atoms and purple nitrogen atoms. The two protein structures are completely superimposable with the exception of three loops in the BABIM-BoNT/B-LC structure which are affected by crystal packing. (b) Close-up of BABIM-BoNT/B-LC interactions. (c) BABIM-BoNT/B-LC rotated 90° about the vertical axis to illustrate BABIM binding orientation compared to substrate.

the active-site cleft. The overall structure of the toxin/inhibitor cocrystal structure was found to be very similar to the synaptobrevin-bound structure recently described (rmsd 1.2 Å) (Figure 1a).[10] Minor differences in loop orientations can be attributed to crystal-packing interactions that result from the 5 Å smaller *c* axis of the unit cell. BABIM binds by displacing the water ligand of the zinc ion and chelating the zinc through one of its deprotonated basic amidinium groups, as well as through hydrogen-bonding interactions between the bis-imidazoyl moieties of BABIM and Glu170. Other weak interactions involve a water-bridged hydrogen bond between Tyr53, Glu168, and the imidazoyl nitrogen of BABIM (Figure 1b). The length of BABIM is directed along the active-site cleft in the substrate C-terminal direction. The overall curvature of BABIM closely resembles the corresponding region of the C-terminal Sb2 product (Figure 1c). However, BABIM forms interactions with one side of the active-site cleft while the C-terminal Sb2 product binds in the center of the cleft. The toxin residues that interact with BABIM are not involved in any interactions with the Sb2 product. The structure indicates that BoNT/B-LC binds BABIM quite differently than the serine proteases. This unique binding mode is likely due to the presence of only one labile zinc ligand in BoNT/B-LC, whereas the bis-imidazoyl moiety requires bi-dentate binding to a zinc ion. (Coordinates deposited in the Protein Data Bank under accession code 1FQH.)

The 1.6 μM affinity of BABIM for BoNT/B-LC is surprising in light of its orientation in the toxin active site. The amidinium group is not a strong zinc chelator, and there are relatively few reinforcing interactions to peptide binding sites. Moreover, the amidinium group has a pK_a = 10; therefore, unless enzyme interactions alter its base strength, only 0.1% of the potential ligand is in the neutral form needed to interact with active-site zinc. The sole inhibitory effect of BABIM seems to be blocking access of the substrate to the zinc ion. Development of higher-affinity, higher-specificity inhibitors should be possible when functionalities that bind to the zinc at physiological pH and to the protease subsites associated with substrate affinity are incorporated.

With a K_i = 1.6 μM, but a molecular weight of only 332, BABIM is a promising lead compound for inhibition of neurotoxins of the *Clostridium* neurotoxin protease family.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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