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A Single-Domain Llama Antibody Potently Inhibits the Enzymatic Activity of Botulinum Neurotoxin by Binding to the Non-Catalytic α-Exosite Binding Region

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Ingestion or inhalation of botulinum neurotoxin (BoNT) results in botulism, a severe and frequently fatal disease. Current treatments rely on antitoxins, which, while effective, cannot reverse symptoms once BoNT has entered the neuron. For treatments that can reverse intoxication, interest has focused on developing inhibitors of the enzymatic BoNT light chain (BoNT Lc). Such inhibitors typically mimic substrate and bind in or around the substrate cleavage pocket. To explore the full range of binding sites for serotype A light chain (BoNT/A Lc) inhibitors, we created a library of non-immune llama single-domain VHH (camelid heavy-chain variable region derived from heavy-chain-only antibody) antibodies displayed on the surface of the yeast *Saccharomyces cerevisiae*. Library selection on BoNT/A Lc yielded 15 yeast-displayed VHH with equilibrium dissociation constants (K_d) from 230 to 0.03 nM measured by flow cytometry. Eight of 15 VHH inhibited the cleavage of substrate SNAP25 (synaptosome-associated protein of 25,000 Da) by BoNT/A Lc. The most potent VHH (Aa1) had a solution K_d for BoNT/A Lc of 1.47×10^{-10} M and an IC_{50} (50% inhibitory concentration) of 4.7×10^{-10} M and was resistant to heat denaturation and reducing conditions. To understand the mechanism by which Aa1 inhibited catalysis, we solved the X-ray crystal structure of the BoNT/A Lc–Aa1 VHH complex at 2.6 Å resolution. The structure reveals that the Aa1 VHH binds in the α-exosite of the BoNT/A Lc, far from the active site for catalysis. The study validates the utility of non-immune llama VHH libraries as a source of enzyme inhibitors and identifies the BoNT/A Lc α-exosite as a target for inhibitor development.

Keywords: botulinum neurotoxin type A; llama VHH; single-domain antibody; α-exosite; naïve yeast-displayed library

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Abbreviations used: BoNT, botulinum neurotoxin; BoNT/A, botulinum neurotoxin serotype A; BoNT/A Lc, botulinum neurotoxin serotype A light chain residues 1–448; BoNT/A Lc_{425}, truncated BoNT/A Lc containing residues 1–425; CDR, complementarity-determining region; FACS, fluorescence-activated cell sorting; FRET, fluorescence resonance energy transfer; Hc, the C-terminal portion of the botulinum neurotoxin heavy chain; IC_{50}, 50% inhibitory concentration; IMAC, immobilized metal affinity chromatography; mAb, monoclonal antibody; scFv, single-chain format of antibody variable regions; SD-CAA, selective growth dextrose casamino acids media; SNAP25, synaptosome-associated protein of 25,000 Da; VH, heavy-chain variable region; VHH, camelid heavy-chain variable region derived from heavy-chain-only antibody; YsCsY, cyan fluorescent protein and yellow fluorescent protein connected through SNAP25 residues 141–206; YFP, yellow fluorescent protein; GST, glutathione *S*-transferase; PDB, Protein Data Bank.

Introduction

Human botulism is caused by *Clostridium botulinum* neurotoxin (BoNT) serotypes A, B, E, and F and is characterized by flaccid muscle paralysis. The illness, when not immediately fatal, requires prolonged hospitalization in an intensive care unit. Besides causing naturally occurring botulism, BoNTs are also classified by the Centers for Disease Control and Prevention as one of the six highest-risk threat agents for bioterrorism.[1] The symptoms of botulism are caused by BoNT,[2] the most poisonous protein known.[3] The crystal structure of BoNT[4] shows three functional domains composed of a light-chain segment and two heavy-chain segments.[4–6] The C-terminal portion of the heavy chain (Hc) is the cell binding domain, which docks the toxin to ganglioside receptors and a protein receptor (or protein receptors) on presynaptic neurons, resulting in toxin endocytosis.[7–9] The translocation domain at the N-terminal portion of the heavy chain (Hn) mediates escape of the toxin light chain (Lc) from the endosome.[10] Depending on serotype, the Lc cleaves one or more members of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex of proteins involved in synaptic vesicle docking, thereby inhibiting neurotransmitter release.[11,12]

The multi-domain structure of BoNT and its mechanism of action provide a number of ways to prevent and treat botulism. The mainstay of treatment for botulism is antitoxin.[13] Antibody products, such as equine antitoxin and human botulism immunoglobulin, are used to treat adult[14,15] and infant botulism,[16] respectively. Antitoxin appears to work primarily by clearing toxin from the circulation before it can accumulate inside the neuron[17] but can also prevent BoNT entry into neurons by binding to the Hc.[18] In addition, antibody may be able to inhibit translocation and catalysis by binding to the Hn and/or Lc, riding into the cell on BoNT, and then interfering with the function of these domains.[10,19] The recent visualization of the protein and ganglioside receptor binding sites on the BoNT Hc may also permit the design of small-molecule drugs that can block toxin uptake.[20–22]

A limitation of the above therapeutics is that they do not work once the toxin has entered the neuron and, therefore, cannot be used to reverse paralysis. Thus, there is considerable interest in developing inhibitors of the translocation and catalytic domains.[23,24] Since the window to prevent translocation is relatively short, most attention has been focused on molecules that prevent the catalytic domain from cleaving their SNARE substrate. Such inhibitors typically mimic substrate and bind in or around the substrate cleavage pocket.[25,26] The crystal structure of the substrate SNAP25 (synaptosome-associated protein of 25,000 Da) complexed to the BoNT/A Lc showed the extended nature of ligand recognition and identified potential exosites of substrate binding that are away from the catalytic active site.[27] While such exosites have been targeted for inhibitor development,[28,29] no such inhibitors have been reported for BoNT.

To explore the range of binding sites for potential BoNT/A Lc inhibitors, we generated and selected a non-immune camelid (llama) library of single-domain VHH (camelid heavy-chain variable region derived from heavy-chain-only antibody) antibodies for binding to the BoNT/A Lc. Such single-domain antibodies have been postulated as more able to bind into enzymatic cavities, and a number of enzyme inhibitors have been generated after immunizing camelids with enzyme antigens.[30,31] In this work, a number of inhibitory VHH were obtained and a selected complex characterized by X-ray diffraction validated the α-exosite as a viable target for BoNT/A inhibitor development.

Results

Generation and initial characterization of single-domain antibodies to BoNT/A Lc

To generate a panel of single-domain antibodies binding the BoNT/A Lc, we constructed a non-immune llama single-domain library for display on the surface of *Saccharomyces cerevisiae*. Briefly, whole blood was isolated from llamas without prior immunization and RNA prepared. After first-strand cDNA synthesis, llama-specific primers annealing to the VH (heavy-chain variable region) and VHH leader sequence genes and to the CH2 gene were used to PCR amplify the VH and VHH gene repertoires. VHH repertoires were separated from VH repertoires by running the PCR fragments on a gel and excising the smaller band. The VHH gene repertoire was reamplified and cloned into the vector pYD2 for display as a C-terminal fusion to the AgaII protein on the surface of *S. cerevisiae*. After transformation, a library with a size of 6.1×10^7 transformants with a VHH-sized insert was obtained. DNA sequencing of 50 VHH genes picked at random revealed 44 unique sequences, indicating that the library was diverse.

The library was induced to display VHH on yeast surface and incubated with recombinant BoNT/A Lc to generate BoNT/A Lc-specific single-domain antibodies. After staining with anti-BoNT/A Lc monoclonal antibodies (mAbs) and a mAb directed to the C-terminal SV5 epitope tag, yeasts displaying VHH and bound to BoNT/A Lc were flow sorted and collected (Fig. 1). After amplification by growth in liquid culture, surface display was induced and the staining, sorting, and growth cycle were repeated twice more (Fig. 1). After three rounds of sorting, collected yeasts were plated and 48 individual colonies were analyzed for binding to BoNT/A Lc. The VHH gene of each binding clone was sequenced, revealing the presence of 15 unique VHH, two pairs of which (Aa1 and A23, and Aa12 and A10) were clonally related based on the VHH complementarity-determining region 3 (CDR3) sequence (Table 1). The affinity of each of the yeast-