

**ENHANCED SUBSTRATES FOR THE
PROTEASE ACTIVITY OF SEROTYPE A
BOTULINUM NEUROTOXIN**

CROSS-REFERENCE TO RELATED
APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Patent Application No. 61/252,675, filed Oct. 18, 2009, the entire disclosure of which is herein incorporated by reference.

STATEMENT REGARDING FEDERALLY
SPONSORED RESEARCH OR DEVELOPMENT

[0002] This work described in this disclosure was made with government support under the Defense Threat Reduction Agency Research Plan 3.10023_07_RD_B of the Department of Defense. The United States Government has certain rights in the invention.

FIELD

[0003] The field of the disclosure is Botulinum neurotoxins and substrates thereof.

BACKGROUND

[0004] Botulinum neurotoxins (BoNTs) are proteins produced by various strains of *Clostridium botulinum*, *Clostridium butyricum*, and *Clostridium baratii*, and are possibly the most toxic substances known (1-3). There are seven BoNT serotypes, designated A through G, each expressed as a single-chain protein of 150 kDa. *Clostridium botulinum* produces all seven serotypes while the other two strains produce one serotype each. The seven serotypes of botulinum neurotoxin (BoNTs) are zinc metalloproteases that cleave and inactivate proteins critical for neurotransmission. Synaptosomal protein of 25 kDa (SNAP-25) is cleaved by BoNTs A, C, and E, while vesicle-associated membrane protein (VAMP) is the substrate for BoNTs B, D, F, and G. Serotype C also cleaves syntaxin. BoNTs are not only medically useful drugs, but are also potential bioterrorist and biowarfare threat agents.

[0005] In most serotypes the single-chain protein is cleaved by endogenous bacterial protease(s) to yield the dichain molecule, consisting of a heavy chain (100 kDa) and a light chain (50 kDa), covalently linked by a disulfide bond. Cleavage of a single-chain to a dichain form increases toxicity (30). Both chains must be present in the disulfide-linked holotoxin form to cause botulism; i.e., individual chains are not toxic (2-5). BoNTs block the release of acetylcholine from peripheral cholinergic nerve endings. The heavy chain of BoNT binds to receptors on peripheral cholinergic neurons, leading to internalization of the toxin. The light chain is a zinc metalloprotease. The receptor for BoNT A is the SV2 protein (32). The disulfide between the heavy and light chains of BoNT A is reduced when BoNT A enters the cytosol. Upon escape from endosomes into the neuronal cytosol, the light chain of each BoNT serotype cleaves only one peptide bond in its respective substrate. The synaptosomal protein of 25 kDa (SNAP-25) is the substrate for BoNTs A, C, and E. Serotype C also cleaves syntaxin. Vesicle-associated membrane protein (VAMP) is the target of BoNTs B, D, F, and G. Proteolysis at any one of these sites inactivates neurotransmitter exocytosis. Neurotransmitters such as acetylcholine transmit nerve impulses to signal muscles to contract. Therefore, prevention

of neurotransmission leads to the flaccid paralysis of botulism (2, 3). The main forms of botulism are food borne, intestinal, and wound related. BoNT can enter the body by, including but not limited to, inhalation, colonization of the digestive tract, ingesting the toxin from foods, and contamination of a wound. Exposure can occur by, including but not limited to, contaminated food, biowarfare, and cosmetic use of inappropriate amounts of botulinum toxin. The toxin actively passes through the lining of the gut to reach the general circulation.

[0006] In order for vesicle fusion to occur with the neuronal membrane, synaptobrevin, present on the synaptic vesicle, must interact with syntaxin and SNAP-25 on the neuronal membrane. The heavy chain of BoNT A is responsible for cell surface binding and membrane translocation. The light chain dissociates from the heavy chain inside the endosome. The light chains of various serotypes of BoNTs cleave the peptide bonds at locations within synaptobrevin, syntaxin, and SNAP-25, therefore preventing fusion between the vesicle and the neuronal membrane and preventing nerve impulse transmission. (31)

[0007] Despite the extreme toxicity of BoNT, clinicians use BoNT to treat an ever-expanding variety of human diseases where pathological conditions are caused by unregulated exocytosis of acetylcholine (6-8). BoNT is used to treat blepharospasm, strabismus, muscle spasms, migraines, upper motor neuron syndrome, sweating, cervical dystonia, cerebral palsy, urinary incontinence, and for cosmetic uses. In addition, recent results suggest that BoNT might be useful in cancer therapy, where its effects on tumor vascular structure enhanced the efficacy of radiation treatments and chemotherapy (9). Finally, BoNT heavy chain and genetically inactivated holotoxin have been studied as potential intracellular drug carriers targeted specifically to neurons (10-13). Holotoxin refers to the fully active form of the toxin, containing both subunits.

[0008] Unfortunately, BoNT is also a serious biowarfare and bioterrorism threat (1, 14). At present, the only treatment available for patients suffering from systemic botulism is supportive care including administration of specific antibodies to eliminate toxin still in the circulation (antitoxin), and mechanical ventilation, if needed. Recovery can take weeks or months. Because of the large number of serotypes and its use in human medicine, large-scale vaccination of the general population against BoNT is impractical and undesirable. Once inside the neuronal cell, BoNT cannot be neutralized by antibodies. Currently, there are no drugs available that can reach intracellular toxin to reverse or mitigate its effects (1, 7, 15). Because the protease activity of BoNT is required for toxicity, efforts are underway to develop specific inhibitors that might be useful as anti-botulinum drugs, or serve as model compounds for effective drug development (16-23).

[0009] FRET substrates for botulinum protease activity have previously been reported (U.S. Pat. Nos. 6,504,006; 6,762,280; 7,034,107; and 7,157,553). Progress in the identification of potential anti-botulinum drugs would be expedited by the availability of substrates suitable not only for high-throughput assays but also for kinetic and mechanistic studies. The present disclosure reports such substrates for BoNT A protease activity, and demonstrates their superiority compared to other substrates.