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**SMALL MOLECULES AND A  
PHARMACOPHORE MODEL FOR  
INHIBITION OF BOTULINUM TOXIN AND  
METHODS OF MAKING AND USING  
THEREOF**

CROSS REFERENCE TO RELATED  
APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 60/501,243, filed 8 Sep. 2003, listing Sina Bavari and James J. Schmidt, as joint inventors, which is herein incorporated by reference in its entirety.

ACKNOWLEDGMENT OF GOVERNMENT  
SUPPORT

This invention was made by employees of the United States Army. The government has rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention generally relates to inhibitors of Botulinum neurotoxin A metalloprotease activity.

2. Description of the Related Art

Botulinum neurotoxins (BoNTs) are produced by spore forming anaerobic bacteria *Clostridium botulinum*, and are among the most lethal of biological poisons. See Schmidt & Stafford (2003) Appl. Environ. Microbiol. 69:297-303; and Kessler & Benecke (1997) Neurotoxicology 18:761-770. Seven immunologically distinct BoNT serotypes (designated A-G) have been identified. See Simpson, L. L. (1989) BOTULINUM NEUROTOXIN AND TETANUS TOXIN, Academic Press, New York. Accidental exposure to BoNTs, for example, through contaminated food, can result in life threatening flaccid paralysis. See Shapiro, et al. (1998) Ann. Intern. Med. 129: 221-228. Furthermore, BoNTs have been weaponized in highly toxic aerosol form, and consequently pose a significant threat to both to civilian and military populations. See Franz, et al. (1997) JAMA 278:399-411; and Amon, et al. (2001) JAMA 285:1059-1070. As a result, there is an urgent need for therapeutic countermeasures against BoNTs. See Goodnough, et al. (2002) FEBS Lett. 513:163-168.

BoNT is secreted as a holotoxin composed of two peptide chains that are linked by a disulfide bridge. See Lacy & Stevens (1999) J. Mol. Biol. 291:1091-1104. The heavy chain is responsible for: (1) targeting and binding to surface receptors on nerve terminals; (2) translocation into the neuronal cytosol via the formation of a low pH endosome; and (3) protecting the substrate binding cleft of the light chain prior to neuronal internalization. See Turton, et al. (2002) Trends Biochem. Sci. 27:552-558; and Singh, B. R. (2000) Nat. Struct. Biol. 7 (2000) 617-619. The light chain, which dissociates from the heavy chain in the low endosomal pH, is released into the cytosol where it acts as a zinc metalloprotease that cleaves soluble NSF-attachment protein receptor (SNARE) proteins: synaptosomal-associated protein of 25 kDa (SNAP-25), synaptobrevin, and syntaxin. BoNT serotypes A, C, and E cleave SNAP-25; serotypes B, D, F, and G cleave synaptobrevin; and serotype C can also use syntaxin as substrate. See Binz, et al. (1994) J. Biol. Chem. 269:1617-1620; Schiavo, et al. (1992) Nature 359:832-835; Schiavo, et al. (1993a) J. Biol. Chem. 268:23784-23787; Schiavo, et al. (1993c) J. Biol. Chem. 268:11516-11519; Schiavo, et al. (1993b) J. Biol. Chem. 269:20213-20216; and Blasi, et al. (1993b) EMBO J. 12:4821-4828. Without functional SNARE

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complexes, acetylcholine is not released into neuromuscular junctions, thereby leading to paralysis.

Research to identify peptide and small molecule inhibitors of BoNT serotype A (BoNT/A) has targeted both holotoxin translocation and light chain (BoNT/A LC) metalloprotease activity. Sheridan et al. and Deshpande et al. have shown that a number of antimalarial agents interfere with BoNT/A translocation into nerve cytoplasm. See Sheridan, et al. (1997) Toxicol 35:1439-1451; and Deshpande, et al. (1997) Toxicol 35:433-445.

Specifically, it has been shown that several antimalarial compounds act subsequent to toxin binding to cell-surface receptors, and it has been hypothesized that these agents inhibit BoNT/A cytosol entry by raising endosomal pH (an endosomal pH of 5.5 or lower is needed for release into the cytoplasm). Hayden et al. have found that BoNT/A LC is inhibited by mM concentrations of known protease inhibitors: captopril, lisinopril, and enalapril. See Hayden, et al. (2003) J. Appl. Toxicol. 23:1-7. In the same study, it was also reported that a number of short peptides, from specific "hinge" libraries, inhibit BoNT/A LC activity by as much as 51% at concentrations as low as 0.5  $\mu$ M. Using a chromatographic method, Schmidt et al. identified the peptide motif CRATKML as a potent inhibitor. See Schmidt, et al. (1998) FEBS Lett. 435:61-64. In a subsequent study, the Cys residue of CRATKML was replaced with thiol containing organic moieties, and it was found that a 2-mercapto-3-phenylpropionyl containing derivative was the most effective ( $K_i=0.3 \mu$ M). See Schmidt & Stafford (2002) FEBS Lett. 532:423-426.

Unfortunately, no small molecule (non-peptidic) inhibitors of BoNT/A LC metalloprotease activity, which are effective in the low  $\mu$ M range, have been reported.

Thus, a need exists for inhibitors of BoNT/A LC metalloprotease activity.

SUMMARY OF THE INVENTION

The present invention generally relates to compounds and pharmacophore models that inhibit BoNT/A LC metalloprotease activity.

In some embodiments, the present invention provides a pharmacophore model for inhibiting Botulinum neurotoxin A metalloprotease activity which comprises a first plane A, a second plane B, a first hydrophobic moiety C, a second hydrophobic moiety D and a positive ionizable substituent E. The pharmacophore model may further comprise a heteroatom in the first plane A. In some embodiments, the distance between the center of the first plane A and the center of the second plane B is about 6.5 to about 9.5 Å. In some embodiments, the distance between the center of the first hydrophobic moiety C and the center of the second hydrophobic moiety D is about 8.0 to about 16.0 Å. In some embodiments, the distance between the center of the first plane to the center of the first hydrophobic moiety C is about 3.0 to about 5.0 Å. In some embodiments, the distance between the center of the second plane to the center of the second hydrophobic moiety C is about 3.0 to about 5.0 Å. In some embodiments, the distance between the center of the first plane to the center of the positive ionizable substituent is about 6.5 to about 9.5 Å. In some embodiments, one or both of the planes comprise a biaryl group or a triaryl group. In some preferred embodiments, the biaryl group is selected from the group consisting of naphthalene, quinoline, isoquinoline, benzofuran, indole, quinazoline, quinoxaline, naphthyridine, phthalazine, and purine. In some preferred embodiments, the triacyl group is selected from the group consisting of acridine, phenazine,