Molecular modelling of botulinum neurotoxin serotype A metalloprotease inhibitors

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Abstract: Botulinum neurotoxins are the most potent neurotoxins known and are ranked by the CDC as category A bioterrorist agents. There is an urgent need to discover and develop small molecule inhibitors as countermeasures for botulinum neurotoxin intoxication. In this report, we summarise our molecular modelling results which contribute to the SAR development and binding site identification of small molecule inhibitors of BoNT/A metalloprotease.

Keywords: botulinum neurotoxins; molecular modelling; metalloprotease; BoNT inhibitor.

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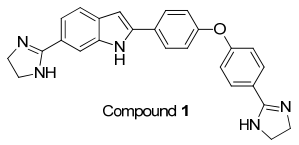
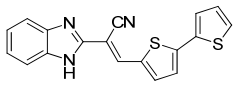
Botulinum neurotoxins (BoNTs), secreted by bacterial *clostridia* species *botulinum*, *baratii* and *butyricum*, are the most potent neurotoxins known and are ranked by the CDC as category A bioterrorist agents. BoNTs are easily produced and may be delivered via an aerosol route. Consequently, these toxins represent a serious threat to both military

personnel and civilians. There is an urgent need to identify and develop low molecular weight, non-peptidic BoNT inhibitors that will serve as both prophylactic agents and post-exposure 'rescue' therapeutics.

Among the seven serotypes, BoNT/A is the deadliest. The light chain (LC) portion of BoNT/A is a zinc-dependent protease, which constitutes a drug target for antidote discovery.

Two independent high throughput screening campaigns have identified an indole-based bis-amidine inhibitor (NSC 240898) (Burnett et al., 2007) and a benzimidazole acrylonitrile inhibitor (Cardinale et al., 2011) of BoNT/A LC, both of which displayed micromolar potency. Medicinal chemistry optimisation and SAR development have yielded improved and specific BoNT/A LC inhibitors 1 and 2 (see Table 1), with cell-based activity in neuronal cells. Interestingly, compounds 1 and 2 demonstrated different mechanisms of inhibition (Li et al., 2010, 2011). Compound 1 is a competitive BoNT/A LC inhibitor, while compound 2 is a non-competitive, time-dependent inhibitor with respect to substrate (SNAPtide; 17-mer, AA 187–203).

Table 1 *In vitro* enzyme inhibition for compounds 1 and 2.

	 Compound **1**	 Compound **2**
	IC_{50} (μM)	IC_{50} (μM)
BoNT/A LC (HPLC)	9.4	29 (17)[a]
BoNT/B LC (FRET)	> 100	> 100
Anthrax LF	43	> 100
MMP-1, 2, 9	> 100	> 100

Note: [a]The IC_{50} value in brackets was obtained by pre-incubation of inhibitor with BoNT/A LC for 90 minutes; total assay time was 130 minutes.

To rationalise the structure-activity relationships of the indole-based BoNT/A LC inhibitors, we performed molecular modelling studies on several compounds in this series. Consistent with the competitive enzyme kinetics, the indole-based inhibitors appear to tightly bind into the active site of BoNT/A LC. Interestingly, these inhibitors apparently do not directly interact with the catalytic Zn atom, which is also consistent with the finding that the inhibitory activity of the indole-based inhibitors is independent of Zn concentration. The two protonated amidines form hydrogen bonding interactions with Asp370 and Pro239, and the indole-NH is hydrogen-bonded to a main chain carbonyl oxygen atom. Molecular modelling with the indole-based bis-amidines also explains several key SAR findings, such as the following: replacing the indole core with the similarly hydrophobic benzothiophene core maintains and slightly improves potency, while replacing the indole core with basic or hydrophilic benzimidazole cores decreases potency; and replacing the central phenyl ring with pyridine decreases potency. The proposed model also suggests that hydrophilic or solubilising groups can be attached to the amidine moieties to improve solubility of the indole-based inhibitors, while maintaining or even improving *in vitro* potency.

The non-competitive, time-dependent enzyme kinetics displayed by the benzimidazole acrylonitrile BoNT/A LC inhibitor 2 suggests that it may bind to a site,

which is differentiated from the active site, by formation of a covalent bond. We used a molecular modelling approach to identify a proposed inhibitor binding site as well as a nucleophilic amino acid that could be responsible for the generation of a covalent adduct. We identified two cysteine amino acid residues (Cys134 and Cys165) located in BoNT/A LC. Docking of compound 2 into both sites revealed that the best docking mode for 2 in the Cys134 site is not favourable, while the docking mode for compound 2 in the Cys165 site is stabilised by hydrogen bonding interactions between 2 and Arg177. Importantly, the S–C_β distance for the docked compound 2 in the Cys165 site is about 4.3 Å, which renders the nucleophilic Michael addition reaction plausible, without the need to overcome a high energy barrier. The covalent binding mode retains the hydrogen bonding interaction between the CN nitrogen atom and the guanine functionality of Arg177. Based on the molecular modelling results, we propose that Cys165 is the preferred nucleophilic amino acid for covalent modification of compound 2, and that a Michael addition occurs between the sulfhydryl group of Cys165 and the acrylonitrile functionality of compound 5 to produce persistent inactivation of BoNT/A LC. We have termed the Cys165 site as the ϒ-exosite, to supplement the known substrate SNAP-25 α- and β-exosites.

In summary, we have used molecular modelling techniques to contribute to the SAR development and binding site identification of small molecule inhibitors of BoNT/A LC.

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