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Pharmacophore-guided lead optimization: The rational design of a non-zinc coordinating, sub-micromolar inhibitor of the botulinum neurotoxin serotype a metalloprotease

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ABSTRACT

Botulinum neurotoxins, responsible for the neuroparalytic syndrome botulism, are the deadliest of known biological toxins. The work described in this study was based on a three-zone pharmacophore model for botulinum neurotoxin serotype A light chain inhibition. Specifically, the pharmacophore defined a separation between the overlaps of several different, non-zinc(II)-coordinating small molecule chemotypes, enabling the design and synthesis of a new structural hybrid possessing a K_i = 600 nM (±100 nM).

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Botulinum neurotoxins (BoNTs) are secreted by anaerobic, spore forming bacteria within the genus *Clostridium*.[1] Structurally, the toxins are composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) that are covalently linked through a disulfide bridge.[2,3] The HC component binds to pre-synaptic membrane receptors at neuromuscular junctions and facilitates toxin internalization.[1] The LC component, which is liberated from the HC via the intracellular reduction of the disulfide linker, is a zinc (Zn(II)) metalloprotease that cleaves components of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) protein complex (composed of SNAP-25 (synaptosomal-associated protein of 25 kDa), VAMP (vesicle-associated membrane protein, also referred to as synaptobrevin), and syntaxin).[4,5] Of the seven known BoNT serotypes (classified A–G), A and E cleave SNAP-25,[6,7] serotypes B, D, F, and G cleave VAMP,[8–11] and serotype C1 cleaves both SNAP-25 and syntaxin.[12] Botulinum neurotoxin mediated proteolysis of SNARE proteins inhibits the exocytosis of acetylcholine into neuromuscular junctions.[1] This in turn results in life threatening flaccid paralysis.

Due to the lethality of BoNTs (e.g., the lethal intravenous dose of BoNT serotype A in humans is 1 ng kg^{-1})[1] and their potential use as bioweapons,[13,14] there is an urgent need for the identification and development of small molecule, non-peptidic, inhibitors (SMNPIs) to counter the toxins' metalloprotease activity—post intoxication; currently available antitoxin vaccines[13] are not effective after neuronal internalization of the toxin. At this time, the majority of such research has focused on the BoNT serotype A LC (BoNT/A LC), as it induces long-term neuroparalysis[15] (i.e., months) and has been responsible for the majority of reported BoNT poisonings.[13] Yet, of the described SMNPIs of the BoNT/A LC,[16–26] only Zn(II)-coordinating *o*,*p*-dichlorocinnamic hydroxamate is reported to possess an IC_{50} < 0.5 μM;[16] however, when this compound was tested in our HPLC-based assay, it was significantly less potent, with an IC_{50} > 29 μM.[17] Moreover, in a subsequent publication by Eubanks et al.,[21] this molecule was reported to be neurotoxic and displayed poor in vivo activity.

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In contrast to SMNPIs incorporating a relatively unselective Zn(II)-coordinating moiety, such as a hydroxamate,[16,20,24,26] our research has focused on the pharmacophore-based identification and development of non-Zn(II)-coordinating SMNPIs.[17–19,25] In particular, we have used three-dimensional search queries derived from our gas phase pharmacophore for BoNT/A LC inhibition. This model relies solely on comparisons of the structures of the small molecules and their accompanying SAR (structure activity relationships), and has resulted in the identification of a variety of non-Zn(II)-coordinating SMNPIs, including 4-amino-7-chloroquinoline (4,7-ACQ) conjugates[18,19,25], multi-heterocyclic dicationic compounds[17,19], and a rigid diazachrysene chemotype.[27] Moreover, as novel SMNPI chemotypes are identified, they are used to further refine the model, thereby improving its ability to guide SMNPI identification and optimization. As an example, we recently reported a refined 3-Zone gas-phase pharmacophore (Fig. 1a) that was generated based on the separation of different SMNPI chemotype overlays.[27] In the same study, we validated this pharmacophore via the discovery a new terephthalamide-based SMNPI (NSC 104999).[27]

The identification of a third pharmacophore zone for inhibitor occupancy is of significant importance to our research program given the fact that we have been unable to improve the potency of our lead for therapeutic development, NSC 240898,[17] solely within pharmacophore Zones 1 and 2 (Fig. 1b). Specifically, we recently reported the synthesis of fourteen congeners of this SMNPI that were generated to explore the possibility of improving its affinity for the BoNT/A LC substrate binding cleft.[28] However, only one derivative, possessing a conservative sulfur replacement of the parent SMNPI's ether oxygen, provided equipotent inhibition.[28] In contrast, the substitution of functional groups on the NSC 240898 central aromatic ring, the incorporation of additional heteroatoms in the arene moiety, and the replacement and/or rearrangement of the inhibitor's two amidine functions, all resulted in less potent derivatives.[28] Thus, in light of the fact that we have been unable to synthetically optimize inhibitory potency within Zones 1 and 2 for this scaffold, our optimization strategy was altered to focus on the incorporation of a Zone 3 component.

The design of an analog of NSC 240898 incorporating a Zone 3 component relied on the previous hypothesis that 4,7-ACQ-based SMNPI Q2-15 (IC_{50} = 11.3 μM (±4.7 μM), and 60% inhibition at 20 uM conc.[19]) occupies pharmacophore Zones 2 and 3 (Fig. 1c).[27] Hence, by overlaying these two SMNPIs (i.e., NSC 240898 and Q2-15) within the context of the 3-Zone pharmacophore, a potential 'hybrid' 3-Zone inhibitor retaining the entire structure of NSC 240898, but with a 4,7-ACQ moiety tethered via a propyl linker, is a plausible synthetic candidate (Fig. 1d). However, because NSC 240898 is not symmetrical, and meets the criteria of pharmacophore Zones 1 and 2 in two orientations (Fig. 1b), two designs were possible (Fig. 1d). In the first, a 4,7-ACQ substructure is tethered to the amidine substituent located on the 6 position of the NSC 240898 indole component. In the second, the 4,7-ACQ is tethered to the amidine located on the 4 position of NSC 240898's terminal phenyl component.

A straightforward method to simultaneously prepare designs **1** and **2** (Fig. 1d) was the preparation of a regioisomer mixture (Scheme 1). In brief, 1 equiv of NSC 240898 (the synthesis of which has been described[29]) was coupled with **4**, which was prepared via the nucleophilic substitution of **3** with propane-1,3-diamine. The reaction was carried out for 30 min in a monomode microwave reactor at 150 °C, and RP-HPLC purification afforded a 4:1 mixture of TFA salts **1** and **2**.

In vitro testing of **1** and **2** in our HPLC-based assay[17,19,30–34] resulted in 95% BoNT/A LC inhibition (at 20 μM conc. SMNPI). A more

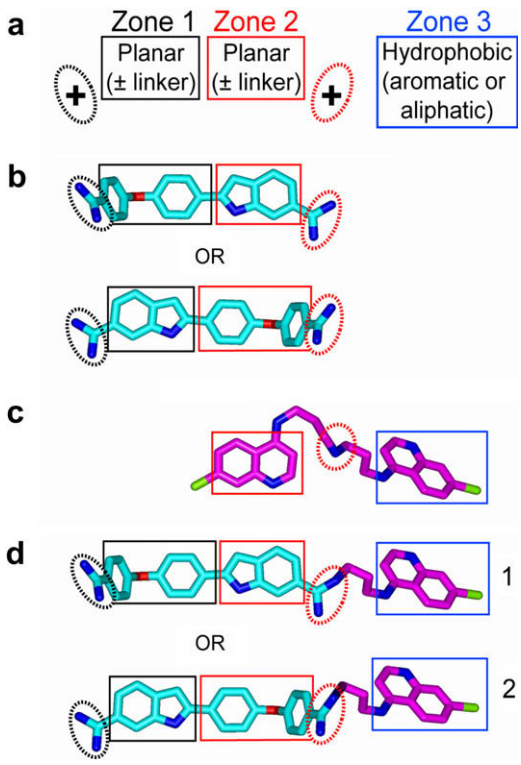
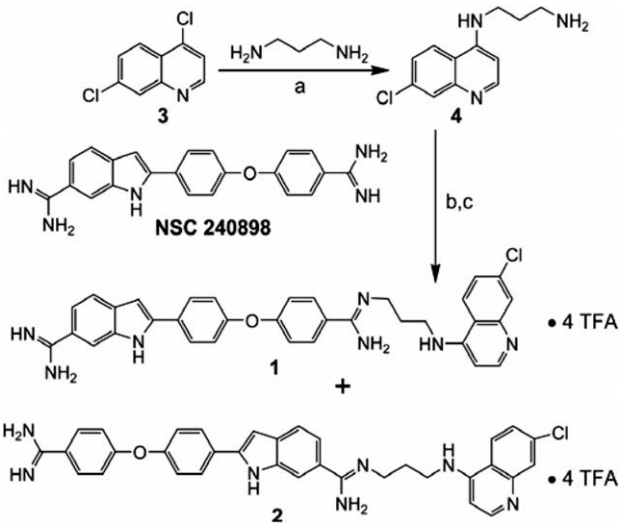


Figure 1. The 3-Zone pharmacophore for BoNT/A LC inhibition provided the design strategy for regioisomers **1** and **2**. (a) The 3-Zone pharmacophore. Zone 1 (black text and shapes) consists of a cationic component (black oval) and a planar component (black rectangle). This Zone may also contain a component that links it to Zone 2. Zone 2 (red shapes) consists of a cationic component (red oval), a planar component (red rectangle), and may also possess a component that links it to Zone 1. Note, while either Zone 1 or Zone 2 may possess the linker component, the Zones cannot simultaneously possess linker components. Zone 3 (blue rectangle) consists of a hydrophobic component that may be aromatic or aliphatic. (b) The previously described SMNPI NSC 240898[17] (cyan carbons, red oxygen, and blue nitrogens) fits equally well within Zones 1 and 2 of the pharmacophore with two orientations. (c) The previously described SMNPI Q2-15[19] (magenta carbons, blue nitrogens, and light green chlorines) occupies Zone 2 and Zone 3 of the pharmacophore.[27] (d) 3-Zone regioisomer designs **1** and **2**. In each case the Zone 3 component of Q2-15 (magenta carbons) is coupled to the Zone 2 amidine component of NSC 240898 (cyan carbons) via a propyl bridge. Since NSC 240898 occupies Zones 1 and 2 in two different orientations, two regioisomeric designs are possible.



Scheme 1. Synthesis of regioisomers **1** and **2**. [a]Reagents and conditions: (a) 120–130 °C, 6–8 h. (b) PhSH, EtOH, μW, 150 °C, 30 min. (c) RP-HPLC: MeOH:H_2O (0.1% TFA), 30:70 to 100:0.

detailed analysis of the inhibition kinetics revealed that the regioisomers possess a K_i = 600 nM (±100 nM). To the best of our knowledge, this is the most potent inhibition of the BoNT/A LC provided by a non-Zn(II)-coordinating SMNPI reported to date. Moreover, this result further validates the 3-Zone pharmacophore for BoNT/A LC inhibition (Fig. 1a), and demonstrates the usefulness of this model for guiding the development of more potent SMNPIs. Specifically, the utility of the pharmacophore is evidenced by the fact that coupling NSC 240898, a 2-Zone inhibitor (Fig. 1b) possessing a K_i = 10 μM[17] (IC_{50} = 3.0 μM (±1.4 μM)[28]) with the Zone 3 component of a second, weaker SMNPI, that is, Q2-15 (Fig. 1c) (IC_{50} = 11.3 μM (±4.7 μM)), provides a 3-Zone hybrid (composed of a regioisomer mixture (Fig. 1d)) that is 16.7-fold more potent than NSC 240898.

Future studies will focus on: 1) determining which, if either of the regioisomers (i.e., **1** or **2**) is more potent, 2) defining a comprehensive SAR for optimizing Zone 3 occupancy, and 3) determining the optimal tether for linking the Zone 2 and 3 components.

In conclusion, we have used a 3-Zone pharmacophore for BoNT/A LC inhibition to guide the design of the first non-Zn(II)-coordinating SMNPI of the BoNT/A LC that is active in the sub-μM range. This entailed coupling a lead for therapeutic development, NSC 240898, with a 4,7-ACQ component via a propyl linker. The validation of the 3-Zone pharmacophore, supported by the increased potency of the 4:1 mixture of regioisomers **1** and **2**, represents a new paradigm for guiding the synthetic optimization of non-Zn(II)-coordinating SMNPIs of the BoNT/A LC.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.111.

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