Analysis of botulinum neurotoxin serotype A metalloprotease inhibitors: analogs of a chemotype for therapeutic development in the context of a three-zone pharmacophore

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Abstract: Botulinum neurotoxins (BoNTs), and in particular serotype A, are the most poisonous of known biological substances, and are responsible for the flaccid paralysis of the disease state botulism. Because of the extreme toxicity of these enzymes, BoNTs are considered highest priority biothreat agents. To counter BoNT serotype A (BoNT/A) poisoning, the discovery and development of small molecule, drug like inhibitors as post intoxication therapeutic agents is being pursued. Specifically, we are focusing on inhibitors of the BoNT/A light chain (LC) (ie, a metalloprotease) subunit, since such compounds can enter neurons and provide post intoxication protection of the enzyme target substrate. To aid/facilitate this drug development effort, a pharmacophore for inhibition of the BoNT/A LC subunit was previously developed, and is continually being refined via the incorporation of novel and diverse inhibitor chemotypes. Here, we describe several analogs of a promising therapeutic chemotype in the context of the pharmacophore for BoNT/A LC inhibition. Specifically, we describe: 1) the pharmacophoric ‘fits’ of the analogs and how these ‘fits’ rationalize the in vitro inhibitory potencies of the analogs, and 2) pharmacophore refinement via the inclusion of new components from the most potent of the presented analogs.

Keywords: botulinum, neurotoxin, inhibitor, pharmacophore, metalloprotease, biothreat

Introduction

Botulinum neurotoxins (BoNTs), secreted by bacteria of the genus Clostridia, have been described as both ‘Dr Jekyll and Mr Hyde’ and ‘two-faced’ enzymes.2 On the one hand, very low concentrations of locally injected BoNTs (mainly BoNT/A), and to a lesser degree BoNT serotype B, provide many beneficial medical treatments.3–10 On the other hand, BoNTs are the deadliest of known biological toxins (eg, the lethal dose of BoNT/A in humans is estimated to be 1.0 ng kg$^{-1}$).1 Due to the ease with which BoNTs may be obtained and disseminated, these toxins are categorized among the highest priority, category A, biothreat agents by the Centers for Disease Control and Prevention.11 Hence, there is an ongoing effort to develop small molecule, nonpeptidic, inhibitors (SMNPIs) to counter the muscle paralyzing effects of these toxins post exposure.

There are seven known BoNT serotypes (designated A–G).12–17 Structurally, the active compositions of the enzymes, following proteolytic processing,1 include a heavy chain (HC) subunit and a light chain (LC) subunit. The HC mediates toxin internalization into the cytosol of motor neurons.18,19 The LC subunit is a zinc (II) metalloprotease that cleaves, depending on the serotype, components of the soluble N-ethylmaleimide-sensitive

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factor the SNARE complex in the neuronal cytosol. The cleavage of proteins forming the SNARE complex inhibits acetylcholine release into neuromuscular junctions, which results in the life-threatening flaccid paralysis associated with botulism.

Of the seven BoNT serotypes, A, B, E and F are known to cause botulism in humans, with BoNT/A possessing the longest duration of activity in the neuronal cytosol (eg, from several weeks to months, depending on the severity of the poisoning). The metalloprotease LC of BoNT/A (ie, the BoNT/A LC), cleaves SNARE component synaptosomal associated protein of 25 kDa (SNAP-25), which, as its name implies, is anchored to the intracellular membrane of the motor neuron terminus. Hence, due to its duration of action, and because it causes botulism in humans, the vast majority of research to develop SMNPIs that will counter BoNT intoxication post neuronal internalization, has focused on the BoNT/A LC.

In this study, we analyzed BoNT/A LC SMNPI analogs of a promising chemotype for therapeutic development, namely, 6-carbamimidyl-2-[4-(4-carbamimidylphenoxy) phenyl]indole (SMNPI 1, Table 1), which: 1) possesses the ability to penetrate into the neuronal cytosol, 2) provides dose-dependent protection of SNAP-25 in neurons, 3) is well tolerated by neurons at 40 µM concentration, and 4) is selective for the BoNT/A LC versus other metalloproteases (eg, anthrax lethal factor, the BoNT serotype B LC, human matrix metalloprotease-1 (MMP-1), and human MMP-9 (data not shown)). First, seven analogs of parent SMNPI 1 possessing half maximal inhibitory concentrations (IC₅₀) values < 25 µM were analyzed and their in vitro activities were rationalized based on their pharmacophoric ‘fits’. Second, the most potent of the analogs (SMNPI 2) was compared in three dimensional (3D) space with different chemotypes that were used to develop the latest iteration of the pharmacophore for BoNT/A LC inhibition (ie, the 3-zone pharmacophore). Finally, 3D comparisons between SMNPI 2 and the other chemotypes were used to further refine the pharmacophore for BoNT/A LC inhibition.

Material and methods
SMNPI synthesis
The syntheses employed to generate the presented SMNPIs (Table 1) will be reported in detail elsewhere. The synthesis of 1 has been reported previously. In general, the reported SMNPIs were obtained from either mononitrile or dinitrile intermediates, which were prepared via either Cadogan–Sundberg indole syntheses or Suzuki coupling reactions. SMNPI 2 was synthesized by treating dinitrile 10 with N,N-dimethylmethylenediamine under Pinner reaction conditions (Scheme 1). SMNPIs 3–5 and 7–8 were synthesized from their corresponding dinitrile intermediates (eg, compounds 10 and 15) employing P₂S₅ (Scheme 2). Analogs 6, 11, 13 and 14 were also synthesized employing either P₂S₅ or the Pinner reaction. Amide substituted analogs 9 and 12 were prepared by treatment of the corresponding dinitrile or mononitrile intermediate, respectively, with 4:1 (v/v) TFA/H₂SO₄.

In vitro testing
The FRET-based assay used to determine BoNT/A LC inhibition has been previously described. In brief, small molecule, 20 µM SNAP-25 peptide substrate (residues 187–203) with the sequence SNRTRIDEAN[DnpK]RA [daciaC]RML (Peptides International, Louisville, KY), and 10 ng of BoNT/A LC (List Biological Laboratories, Campbell, CA) were incubated at 37°C for 40 min in the presence of buffer (50 mM HEPES–0.05% Tween, pH 7.4) (final volume = 100 µL). For each assay run, the reaction was terminated using acetic acid (0.5% of the final conc.) before fluorescence measurement of the cleaved substrate (at 485 nm) following excitation at 398 nm employing a Molecular Devices plate reader (Sunnyvale, CA). Half maximal inhibitory concentrations (ie, IC₅₀ values) were calculated via dose-response measurements.

Pharmacophore modeling
All SMNPI overlays (ie, superimpositions and alignments), for the evaluation of SMNPIs within the context of the pharmacophore, were conducted using Insight II (version 2005) software (Accelrys, San Diego, CA). Moreover, SMNPI conformation energy refinements were carried out using the Discover program (Accelrys) (cff91 force field) as a module within Insight II. Conformations of SMNPIs were examined for viability using an intramolecular atom-atom Van der Waals bump cutoff of 0.25 Å. All modeling using Insight II was performed on a Dell Precision 690 workstation (Dell Inc., Round Rock, TX) running Linux Red Hat Enterprise (version 4; Red Hat Inc., Raleigh, NC). Figure 2 was generated using Insight II.

Results and discussion
A promising lead BoNT/A LC SMNPI chemotype for development as a potential therapeutic agent (SMNPI 1, Table 1) was discovered via database mining of the NCI Open Repository, and subsequently, a limited number of
Table 1 Parent SMNPI 1, analogs 2–8 possessing IC_{50} values < 25 µM, and (for comparison) inactive analogs 9–15. The compound components are colored as they ‘fit’ the pharmacophore displayed in Figure 1 (zone-1 components are black and zone-2 components are colored red). Substituents deviating from the pharmacophore are colored green. Non-cationic zone-1 and zone-2 components, resulting in inactive analogs 9–15, are colored burgundy.

<table>
<thead>
<tr>
<th>SMNPI</th>
<th>Structure</th>
<th>In vitro potency (IC_{50}) µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="Structure1" /></td>
<td>11 µM</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Structure2" /></td>
<td>2.5 µM</td>
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<tr>
<td>3</td>
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<td>12.5 µM</td>
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<td>6</td>
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<td>20 µM</td>
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<tr>
<td>7</td>
<td><img src="image" alt="Structure7" /></td>
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<tr>
<td>8</td>
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<td>9</td>
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<td>&gt;100 µM</td>
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<td>13</td>
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(Continued)
analogs were synthesized and reported. Using this SMNPI chemotype (in conjunction with other, structurally different BoNT/A LC SMNPI chemotypes), a gas-phase, 3-zone pharmacophore for BoNT/A LC inhibition was generated (Figure 1). Importantly, the pharmacophore was generated based solely on the 3D overlays of the hydrophilic and sterically complementary components shared by diverse BoNT/A LC SMNPI chemotypes. Additionally, in the same study, the 3-zone pharmacophore was validated via its use to generate a 3D search query that, via 3D database mining, identified a novel BoNT/A LC SMNPI chemotype. Moreover, the 3-zone pharmacophore was subsequently validated by research demonstrating that the synthesis of a designed SMNPI incorporating a zone-3 component produced an SMNPI with nM range inhibitory efficacy. However, the substituent composition necessary for SMNPI optimization within zone-3 has yet to be defined (Figure 1). For example, at this time we know that both aliphatic and aromatic moieties can occupy this zone, but we do not know if other substituents are tolerated or will demonstrate improved zone-3 occupancy. Therefore, to further increase the overall resolution of the pharmacophore, we are constantly incorporating new BoNT/A LC SMNPI chemotypes, as well as analogs of known BoNT/A LC SMNPIs. In this study, a variety of analogs of parent SMNPI 1 (Table 1), a known small molecule chemotype with therapeutic potential, were analyzed.

With regard to 1, this SMNPI occupies only zones-1 and -2 of the 3-zone pharmacophore (Figure 1); however, as indicated above, the addition of a pharmacophore zone-3 component on this chemotype gives an SMNPI with superior inhibitory potency. To evaluate the pharmacophoric ‘fits’ of the analogs of SMNPI 1 (Table 1) presented in this study, the first phase of the research involved examining seven active analogs (ie, those demonstrating IC<sub>50</sub> values &lt;25 µM) for pharmacophoric ‘component’ complementarity, as well as for any novel substitutions that might contribute to the refinement of the current pharmacophore model.

As observed in Table 1, analogs 2–8 all possess the previously described pharmacophore zone-1 and -2 ‘flanking’ cationic substituents that are necessary for low µM range inhibitory potency. However, the nearly equivalent IC<sub>50</sub> values of SMNPIs 1 and 5 indicate that either amidine or imidazole substituents are equally efficient with respect to inhibition of the BoNT/A LC; comparatively, the higher IC<sub>50</sub> value for SMNPI 7 indicates that flanking 1,4,5,6-tetrahydropyrimidines are also tolerated, but are not optimal substituents. In this regard, it is hypothesized that the cationic substituents of SMNPIs 1–8 (Table 1) engage in hydrogen bonds with anionic residues of the BoNT/A LC. Hence, for SMNPI 7, the additional hydrophobic ring methylene units of the 1,4,5,6-tetrahydropyrimidine substituents are hypothesized to interfere with SMNPI: BoNT/A LC binding. This hypothesis provides a rationale for SMNPI 7 being less active than SMNPIs 2–6 (Table 1). Indeed, our hypothesis regarding the decreased inhibitory potency of SMNPI 7 appears to correspond with the fact that reestablishing a hydrogen bond donor on the 5-positions of these six-membered-ring systems (ie, 1,4,5,6-tetrahydropyrimidin-5-ol), to give SMNPI 4, results in an analog with an IC<sub>50</sub> value that is 1.5-fold more potent than parent SMNPI 1 (Table 1).

For comparison, Table 1 also shows analogs 9–15, which are structurally similar to SMNPIs 1–8, but which possess amides, a chlorine, a fluorine, a methoxy, or nitrile functional groups at the same flanking positions of pharmacophore zones-1 and -2; analogs 9–15 are all inactive. This analysis provides indisputable evidence supporting the importance of the zone-1 and -2 cationic components of the pharmacophore (Figure 1) that are found in 1–8.

Analogs 1–8 also possess the zone-1 and -2 planar components that are, as specified by the 3-zone pharmacophore
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However, SMNPI 6 possesses an 'abridged' zone-1 planar component compared to other analogs displayed in Table 1. Specifically, for parent SMNPI 1, zone-1 is composed of two phenyl rings bridged by an oxygen atom. By contrast, the abridged zone-1 planar component of SMNPI 6 (ie, a lone phenyl ring), significantly shortens the length of this analog compared to parent SMNPI 1 (Table 1); this deviation from the pharmacophore rationalizes the decreased potency of SMNPI 6 versus SMNPI 1 (Table 1).

Furthermore, the higher IC\textsubscript{50} value for SMNPI 8 indicates (compared to the inhibitory efficacies of all other SMNPIs in Table 1), that a benzimidazole, while tolerated for this SMNPI chemotype, is not an optimal substitution for an indole as the planar component of pharmacophore zone-2.

Regarding new analog structural components that differ from parent SMNPI 1, and which may be used to refine the pharmacophore model, the inhibitory efficacy of benzothiophene containing SMNPI 3 (Table 1) indicates that either a hydrogen bond donor (ie, the indole nitrogen atom of the parent chemotype 1) or acceptor (the sulfur atom of benzothiophene-based 3) is tolerated as the heteroatom in the planar ring system component of zone-2. However, based on the increased potency of SMNPI 3, which is 1.5 fold more potent than parent SMNPI 1, and 1.8 fold more potent than SMNPI 5 (Table 1), it appears that a hydrogen bond acceptor is preferred at this position in the zone-2 heteroatom-containing ring system. Interestingly, this was also observed for several SMNPIs that were identified at the same time as was SMNPI 1, which possessed zone-1 benzofuranyl heteroatom containing ring systems\textsuperscript{29} versus an indole. Finally, the superior inhibitory efficacy of SMNPI 2 (Table 1), which is 4.4 fold more potent than parent SMNPI 1, indicates that extending two cationic N,N-dimethylaminoethyl substituents from both ends of the zone-1 and zone-2 cationic components is favorable. Importantly, the extended N,N-dimethylaminoethyl substituent on the zone-2 amidine of SMNPI 2 (Table 1) is hypothesized to occupy zone-3 of the 3-zone pharmacophore for BoNT/A LC inhibition, and partially accounts for the improved inhibitory potency of SMNPI 2 compared to either parent SMNPI 1, or any other analogs (ie, SMNPIs 3–8 shown) in Table 1. This hypothesis is analyzed in detail below using 3D superimpositions with structurally diverse BoNT/A LC SMNPIs that were used to develop the 3-zone pharmacophore for BoNT/A LC inhibition.

Comparisons of SMNPIs 1–8 (Table 1) (discussed above) prefaced the second phase of this study: the analysis of how potent SMNPI 2 (Table 1) overlays with other, structurally diverse SMNPIs in the context of the 3-zone pharmacophore, and how the inclusion of SMNPI 2, along with zone-1 information from SMNPI 3, served to refine the pharmacophore.
of data needed to unequivocally justify pharmacophore refinement via the inclusion of a zone-4 component.

Finally, although SMNPI 3 (Table 1) is not shown in the Figure 2b overlay, the inhibitory efficacy of SMNPI 3, which is more potent than parent SMNPI 1, implies that either a hydrogen bond acceptor or a hydrogen bond donor is allowed as the heteroatom in the planar ring component of pharmacophore zone-2. Specifically, SMNPI 3 possesses a zone-2 benzothiophene core (Table 1), versus the indole zone-2 core found in all other SMNPIs in Table 1. Therefore, in Figure 2b, the pharmacophore has been refined by showing that the zone-2 planar ring system may contain either a hydrogen bond donor or a hydrogen bond acceptor.

**Conclusion**

Several analogs of potent BoNT/A LC SMNPI 1 (Table 1), which is a lead chemotype for development as a potential therapeutic agent, were evaluated in the context of a 3-zone pharmacophore for BoNT/A LC inhibition. The most potent SMNPI analog (SMNPI 2, Table 1) was overlaid with other, structurally diverse SMNPIs (Figure 2b), and in combination with SMNPI 3, resulted in refinement of the 3-zone pharmacophore for BoNT/A LC inhibition.

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**Figure 1** The 3-zone pharmacophore for BoNT/A LC inhibition (in two-dimensions). Parent SMNPI 1 is shown as it 'fits' within pharmacophore zone-1 and zone-2.

**Figure 2** Overlay of SMNPIs 1 (Table 1), Q2-15, and NSC 104999 (which was identified via a 3D search query that was based on the 3-zone pharmacophore) within the context of the 3-zone pharmacophore for BoNT/A LC inhibition. The Figure 2a overlays are displayed to preface the SMNPI overlays shown in Figure 2b. Figure 2b displays the 3D overlays of SMNPIs 2 (Table 1), NSC 104999, and Q2-15 in the context of the 3-zone pharmacophore. Not surprisingly, this overlay clearly shows that the extended N,N-dimethylaminoethyl substituent on the zone-2 amidine of SMNPI 2 can occupy pharmacophore zone-3. Interestingly, the cationic tertiary nitrogen of SMNPI 2 superimposes closely with the weakly basic 4-amino functionality of the Q2-15 4-amino-7-chloro-quinoline component that occupies zone-3 (Figure 2b). At the same time, the methylene units of the N,N-dimethylaminoethyl substituent extending from the zone-2 amidine of SMNPI 2 superimpose well with the N-butyl moiety methylene units of NSC 104999 that occupy zone-3. Hence, the overlays shown in Figure 2b refine the 3-zone pharmacophore for BoNT/A LC inhibition by indicating that zone-3 may be occupied by not only aromatic and hydrophobic-aliphatic moieties, but also by an aliphatic-cationic moiety (ie, the tertiary amine of SMNPI 2).

The overlay of SMNPI 2 with SMNPI NSC 104999 in Figure 2b also indicates the possibility of a new pharmacophore zone-4 as an extension from zone-1. Previously, it was speculated that there may be additional binding surface complementarity for moieties extending from SMNPI zone-1. The close superimposition of the N-butyl moiety extending from the zone-1 aromatic component of NSC 104999 and the N,N-dimethylaminoethyl substituent extending from the zone-1 amidine of SMNPI 2 provides additional evidence supporting this hypothesis. However, more SMNPI testing is required to accumulate the necessary quantity and quality of data needed to unequivocally justify pharmacophore refinement via the inclusion of a zone-4 component.

Finally, although SMNPI 3 (Table 1) is not shown in the Figure 2b overlay, the inhibitory efficacy of SMNPI 3, which is more potent than parent SMNPI 1, implies that either a hydrogen bond acceptor or a hydrogen bond donor is allowed as the heteroatom in the planar ring component of pharmacophore zone-2. Specifically, SMNPI 3 possesses a zone-2 benzothiophene core (Table 1), versus the indole zone-2 core found in all other SMNPIs in Table 1. Therefore, in Figure 2b, the pharmacophore has been refined by showing that the zone-2 planar ring system may contain either a hydrogen bond donor or a hydrogen bond acceptor.

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Disclosures
The authors report no conflicts of interest in this work.

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