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Three-Dimensional Database Mining Identifies a Unique Chemotype that Unites Structurally Diverse Botulinum Neurotoxin Serotype A Inhibitors in a Three-Zone Pharmacophore

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A search query consisting of two aromatic centers and two cationic centers was defined based on previously identified small molecule inhibitors of the botulinum neurotoxin serotype A light chain (BoNT/A LC) and used to mine the National Cancer Institute Open Repository. Ten small molecule hits were identified, and upon testing, three demonstrated inhibitory activity. Of these, one was structurally unique, possessing a rigid diazachrysene scaffold. The steric limitations of the diazachrysene imposed a separation between the overlaps of previously identified inhibitors, revealing an extended binding mode. As a result, the pharmacophore for BoNT/A LC inhibition has been modified to encompass three zones. To demonstrate the utility of this model, a novel three-zone inhibitor was mined and its activity was confirmed.

Introduction

Botulinum neurotoxins (BoNTs), secreted by spore forming, anaerobic bacteria *Clostridium botulinum*, *C. argentinense*, *C. butyricum*, and *C. baratii*, are responsible for the neuroparalysis associated with botulism. Structurally, BoNTs are composed of a heavy chain (HC) and a light chain (LC), which are linked by a disulfide bridge.[1,2] The HC component binds to surface receptors on neurons and mediates internalization. Once inside neurons, the LC (a zinc (Zn) metalloprotease) cleaves soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which mediate acetylcholine release into neuromuscular junctions.[3] Of the seven known BoNT serotypes, A and E cleave SNAP-25 (synaptosomal-associated protein of 25 kDa),[4] B, D, F, and G cleave VAMP (vesicle-associated membrane protein, also referred to as synaptobrevin),[5–8] and C cleaves both SNAP-25 and syntaxin.[9]

In localized doses, the neuroparalysis resulting from BoNT poisoning: 1) alleviates a range of medical conditions (for example, spastic muscle movement, chronic pain, and anal fissure)[10–19] and 2) is an effective cosmetic treatment for wrinkle reduction.[20–22] On the other hand, BoNTs, as the most lethal of biological substances (for example, it is estimated that the lethal dose of BoNT/A for humans is between 1–5 ng kg^{-1}[23]), are considered among the highest priority biothreat agents by the Centers for Disease Control and Prevention.[24] In addition to being well suited for contaminating consumables (that is, food and liquids), BoNTs can also be delivered by aerosol route.[25]

Neither antitoxin vaccines[26] nor experimental antibodies[23] can counter BoNT metalloprotease activity in the neuronal cytosol. Hence, the only postintoxication treatment currently available, as the paralysis of thoracic muscles ensues, is mechanical respiration. Further complicating BoNT poisoning is the longevity of LC metalloprotease activity, which can last for several weeks depending on the serotype (for example, the effects of BoNT/A poisoning may persist from four to six weeks).[27] Consequently, the intense in-patient medical care needed to treat botulism is impractical; in contrast, small molecule, nonpeptidic, inhibitors (SMNPIs) of the LC metallo-

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protease may serve as both postintoxication 'rescue' therapeutics and as prophylactics.

We have previously described structurally diverse, non-Zn coordinating SMNPIs of the BoNT serotype A LC (BoNT/A LC).[28–31] In this study, we describe how an SMNPI that is active in neurons was used to generate a three-dimensional (3D) search query that identified a rigid diazachrysene-based inhibitor, and how the steric limitations imposed by this scaffold provided the basis for the evolution of a refined, three-zone (3-zone) pharmacophore model for BoNT/A LC inhibition. Finally, we describe how the addition of a third zone facilitated the discovery of a novel compound that represents a distinct structural class of SMNPI.

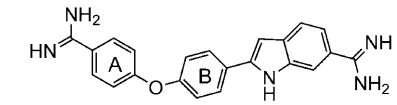
Results and Discussion

At the current stage in the discovery and development of SMNPIs of the BoNT/A LC, there are still many unknowns regarding the steric and chemical requirements necessary for achieving optimal potency. This is evidenced by the fact that only a handful of small molecules with IC_{50} and/or K_i values <15 μM are described in the literature.[28,29,32–38] Among these, only one compound, Zn coordinating *o,p*-(dichloro)-cinnamic hydroxamate (*o,p*-DCH), is reported to possess an inhibition constant lower than 0.5 μM ($IC_{50}=0.41$ μM); however, when examined in our HPLC-based assay, *o,p*-DCH was found to be significantly less potent, with an IC_{50} value of >29 μM,[28] and in a subsequent publication[37] it was shown to be toxic to neurons and demonstrated poor in vivo protection.

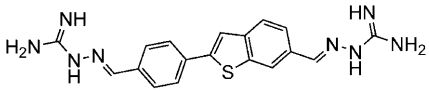
As part of our research program to identify non-Zn coordinating SMNPIs of the BoNT/A LC, we have pursued a parallel strategy that uses only small molecules, and their accompanying structure–activity relationships (SAR), to refine the pharmacophore for BoNT/A LC inhibition[28,31] in gas phase. An integral part of this strategy relies on 3D database mining to identify novel SMNPI chemotypes; possessing a diverse range of inhibitor scaffolds not only increases our understanding of the steric requirements and chemical characteristics necessary to achieve optimal potency, but also provides a variety of chemotypes for potential optimization. This information is of particular importance to our research program given the fact that we have been unable to generate an X-ray co-crystal of our lead candidate for development, NSC 240898, in complex with the BoNT/A LC.[28] Moreover, synthetic chemistry guided by structure-based molecular docking studies has failed to provide an NSC 240898 derivative possessing increased potency (in vitro, data not shown). Given our limited knowledge about the interaction between NSC 240898 and the complementary binding surface of the BoNT/A LC conformation(s) with which it interacts, the study described herein highlights the development of a gas-phase pharmacophore for BoNT/A LC inhibition, and how it facilitated the discovery of novel SMNPIs in the absence of reliable binding site models.

Generation of an internally consistent search query

The search query described in this study was based on NSC 341909[28] ($K_i=3.0$ μM) and NSC 240898[28] (Figure 1), a lead SMNPI for therapeutic development, which displays dose-dependent inhibition of BoNT/A induced SNAP-25 cleavage in neurons (with no toxicity at concentrations as high as 40 μM), and possesses a K_i value of 10 μM[28] ($IC_{50}=3.0$ μM ± 1.4).



NSC 240898 (K_i = 10 μM)



NSC 341909 (K_i = 3.0 μM)

Figure 1. Two-dimensional structures of NSC 240898 and NSC 341909. These previously identified SMNPIs were used to generate the 3D search query for database mining of the NCI Open Repository. For NSC 240898, aromatic rings have been labeled to facilitate further structural description in the Results and Discussion.

As indicated by previous studies,[28,29,31] four key components for inhibitory potency were used to generate the database query. These consisted of two aromatic centers and two flanking cationic centers. The incorporation of specific angle and distance range constraints in the query was balanced to enable both internal consistency and the discovery of novel molecular scaffolds. The final search query and how it maps to conformers of NSC 240898 and NSC 341909 are shown in Figure 2a–c, respectively.

The identification of a novel inhibitor chemotype

Virtual screening of a multiconformational 3D database of the National Cancer Institute (NCI) Open Repository (approximately 270,000 compounds) resulted in the identification of twenty 'hits' that mapped to the search query. Ten were available in sufficient quantities for testing, and of these, three were found to inhibit BoNT/A LC in our HPLC-based assay.[28,29,31,39–43] The 2D structures of the three active compounds and their biological activities are shown in Figure 3. The structures of the three SMNPIs as they map to the search query are shown in Figure 2d–f.

Both NSC 375162 and NSC 377363 (Figure 2) are close congeners of NSCs 341909[28] and NSC 240898 (Figure 1), respectively; in contrast, NSC 328398 (*N,N'*-bis-(3-diethylaminopropyl)-2,8-dimethyl-1,7-diazachrysene-4,10-diamine), which is composed of a central diazachrysene scaffold (Figure 3), constitutes a unique SMNPI chemotype. The structures of the other seven compounds identified by 3D database mining, but which failed to inhibit the BoNT/A LC, are provided in figure 1

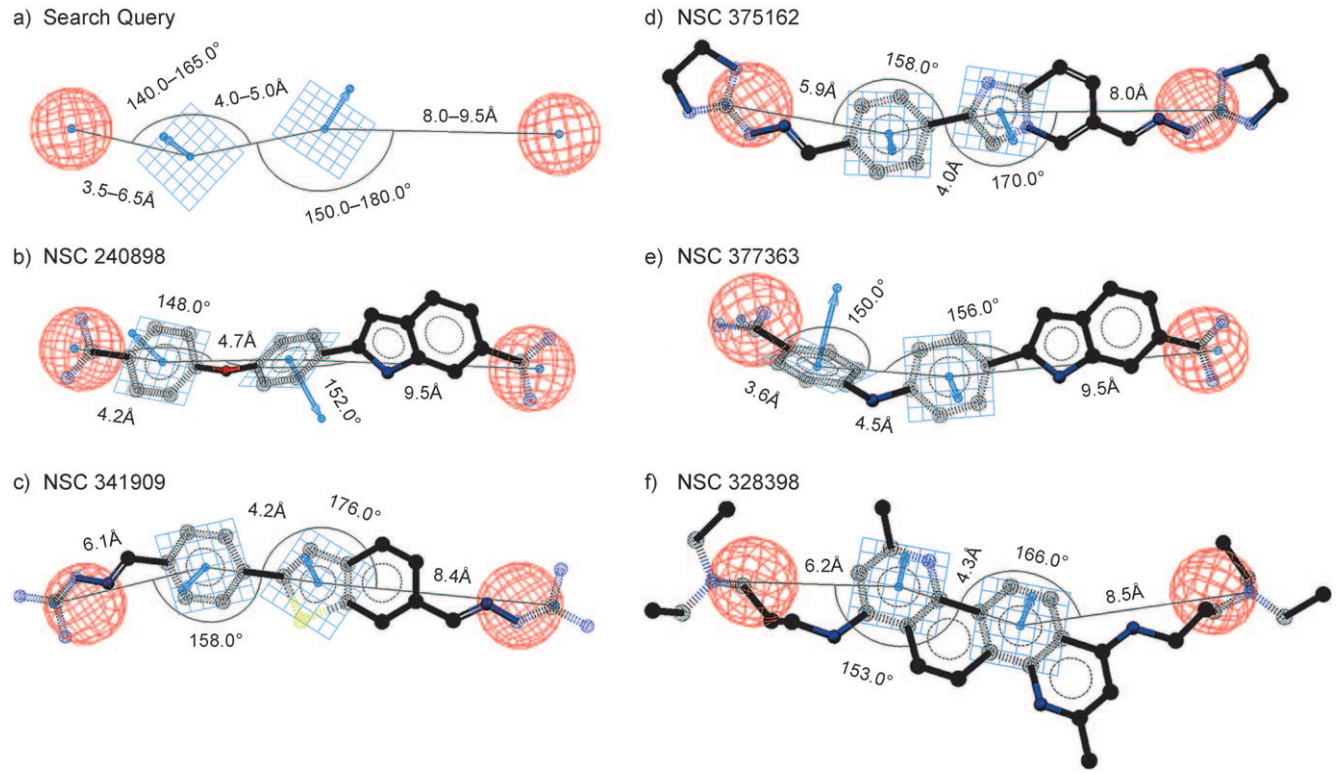


Figure 2. The two-zone pharmacophore search query mapped to SMNPIs: a) The search query with distance and angle constraints. Light blue grids with vectors are aromatic centers and red mesh spheres are cationic centers; b, c, d, e, and f) The search query mapped to NSCs 240898, 341909, 375162, 377363, and 328398, respectively. Carbon atoms are black, nitrogen atoms are blue, oxygen is red, and sulfur is yellow.

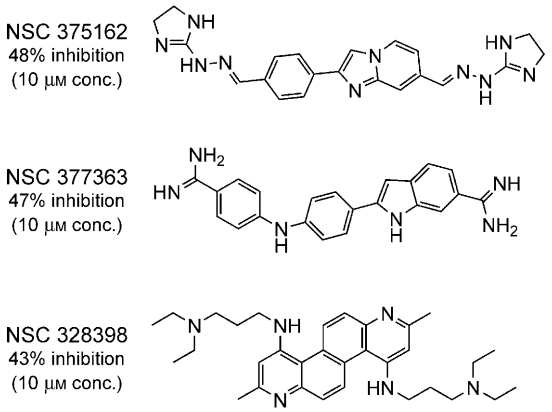


Figure 3. Two-dimensional structures of the three active SMNPIs identified by the search query. The compounds were tested at 10 μM concentrations. The inhibition data is shown graphically in figure 2a of the Supporting Information.

of the Supporting Information. Moreover, by superimposing the search query features of the inactive compounds with those of reference SMNPIs NSC 240898 and NSC 341909, insights into the chemical and steric features hypothesized to be responsible for their inactivity are possible (these structural features are colored red in figure 1 of the Supporting Information. For example, the inactivity of NSC 38283 appears to result from one its sulfonamide moieties, whereas the inactivity of NSC 340844 most likely results from its *N*-oxide components.

To underscore the importance of utilizing 3D structural information to perform our database searches, we conducted a topology-based fingerprint analysis incorporating several functions to illustrate the limitations of these types of methods (see Supporting Information for methodology details). We used Tanimoto, Dice, and Cosine functions to find similar molecules to our reference molecules NSC 240898 and NSC 341909 (these were chosen as they formed the basis of our 3D search query). Using a coefficient of minimum similarity (0.3) we were able to identify only NSC 377363 and NSC 375162 (that is, from our results database consisting of the ten compounds that were tested). These hits (NSC 377363 and NSC 375162) are close congeners of the two reference molecules. Furthermore, we performed the same fingerprint analysis on the NCI Open Repository database and 149 hits were found. Of these, only 80 had sufficient inventory for testing. From those compounds, 22 had been tested previously, with the remaining 58 lacking the minimum pharmacophoric features to warrant testing.

Additionally, we employed a Euclidean distance function to detect similar molecules (30% similarity) based on calculated properties from topology including: Log*P*, molecular weight, number of hydrogen bond donors, number of hydrogen bond acceptors, the number of rotatable bonds, the number of rings, the number of aromatic rings, and the polar surface area using the same two reference molecules (that is, NSC 240898 and NSC 341909). Tested against the results database, NSC 377363, the close congener of NSC 240898, was found. NSC 110682 and NSC 225386 were also hits, and although they

are structurally more diverse than NSC 377363 relative to NSC 240898, both compounds were confirmed to be inactive (see figure 1 in the Supporting Information). Moreover, in contrast to the fingerprint analysis, this method found 80724 hits when run against the NCI Open Repository database, thereby obviating testing in our confirmatory assay.

From these results, we conclude that properties calculated from molecular topology are incapable of producing the results obtained by our 3D search query. This is particularly important given the fact that the 3D search query identified novel SMNPI NSC 328398 from a judicious and manageable set of 20 'hits' (only ten of which were available in sufficient quantities for biological testing).

NSC 328398 is an SMNPI hybrid

Superimposition of NSC 240898 and NSC 328398 clearly demonstrates comparable steric and functional group occupancy in 3D space (Figure 4a). However, unlike NSC 240898, some structural components of NSC 328398 are strikingly similar to those of the previously described SMNPI Q2-15.[31] Figure 5 highlights these similar structural components. In particular, the 4,10-diazachrysene-1,7-diamine substructure of NSC 328398 is comparable to two fused 4-amino-quinolines (ACQ), whereas the aminoalkyl substituents on the 4- and 10-positions of the fused ring system are similar to the aminoalkyl linker of Q2-15 (Figure 5).

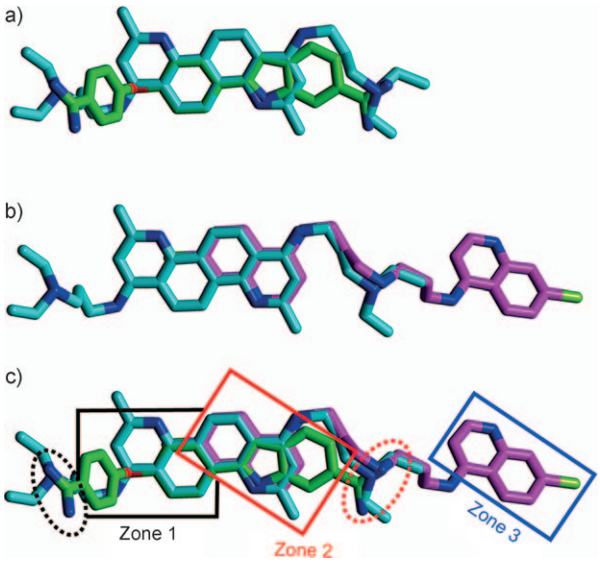


Figure 4. 3D alignments of SMNPIs. Chlorines are light green, nitrogen atoms are blue, and oxygen is red: a) NSC 240898 and NSC 328398 superimposed. NSC 240898 carbon atoms are green and NSC 328398 carbon atoms are cyan; b) NSC 328398 and Q2-15 superimposed. NSC 328398 carbons are cyan and Q2-15 carbons are magenta; c) NSC 240898, NSC 328398, and Q2-15 superimposed. All SMNPI carbon colors are as indicated in a) and b). Based on the alignment, a refined pharmacophore possessing three zones is proposed. Squares indicate planar components and dashed spheres indicate cationic components.

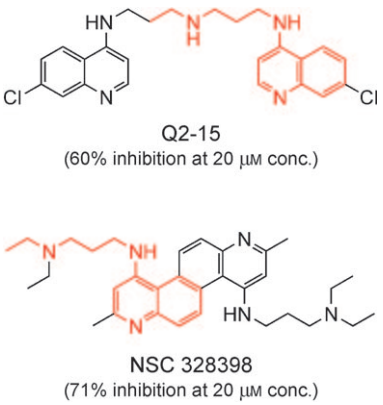


Figure 5. Two-dimensional structures of Q2-15[31] and NSC 328398, with structural similarities colored red.

This analysis led to the superimposition of Q2-15[29,31] and NSC 328398 (in its conformation as it superimposes with NSC 240898) (Figure 4b). Previous SAR indicated that the cationic, secondary amine found in the flexible aminoalkyl linker of Q2-15 (and structurally similar SMNPIs[29,31]) is critically important for inhibition; if this nitrogen is removed and/or replaced activity is essentially lost.[29,31] Hence, one of the NSC 328398 side-chain tertiary amines (the tertiary amine of the 4-position substituent was used) and the Q2-15 secondary amine were superimposed, and served as anchor points for further comparison (Figure 4b). Accordingly, it was clear that the propyl chain on one side of the Q2-15 linker amine possessed the ability to superimpose on the corresponding propyl substituent of NSC 328398. Interestingly, we have previously shown that adding the propylamine component to ACQ, providing N^1-(7-chloroquinolin-4-yl)-propan-1,3-diamine, significantly increases inhibitor potency.[29] Moreover, one of the two ethyl substituents on the NSC 328398 tertiary amine also possesses the ability to superimpose on the Q2-15 propyl chain located on the opposite side of the linker amine (Figure 4b). With the indicated alignments in place, one of the Q2-15 ACQ components oriented so that it corresponded to half the diazachrysene scaffold. As shown in Figure 4b, the superimposed aromatic components of the ACQ and diazachrysene possessed good steric and chemical complementarity.

We next added NSC 240898 to the model (in the same conformation that was superimposed with NSC 328398, described above). The superimposition revealed a third zone of inhibitor occupancy for the second ACQ moiety of Q2-15. Specifically, the alignments in Figure 4c indicate that NSC 240898 and NSC 328398 can occupy the same binding site locations, but that Q2-15 exploits a heretofore unrealized site that can accommodate an ACQ substructure.

Pharmacophore refinement characterized by three zones for inhibitor binding

Until this time, it was hypothesized that NSC 240898 (and similar bis-cationic SMNPIs[28]) and Q2-15 (and other ACQ-based SMNPIs[29,31]) were occupying the same binding site in the

BoNT/A LC substrate cleft,[28,29,31] and the published pharmacophore for BoNT/A LC inhibition reflects this ideology.[28–31] However, based on the discovery of NSC 328398, and because of its unique hybrid structure that organizes NSC 240898 (and similar bis-cationic SMNPIs) and ACQ-based SMNPIs, such as Q2-15, in an extended binding mode, the pharmacophore model was appropriately expanded (Figure 4c).

The refined pharmacophore suggests that all of the inhibitors that we have identified up to this point[28,29,31] occupy only two of three possible binding 'zones.' Specifically, as none of our SMNPIs utilize Zn coordination as part of their binding mode, a third zone of occupancy will provide additional opportunities to increase favorable contacts in the BoNT/A LC substrate binding cleft, thereby increasing binding affinity. This revised hypothesis, referred to as the '3-zone pharmacophore' for BoNT/A LC inhibition (Figure 4c), enables a new classification of previously identified SMNPIs relative to their structural components occupying each zone. Zone 1 is composed of a planar component (for example, NSC 240898 phenyl ring A (see Figure 1 for phenyl ring lettering), a linker (for example, the NSC 240898 phenoxide which incorporates ring B), and one cationic moiety (for example, the NSC 240898 phenyl ring A amidine substituent) (Figure 4c). Zone 2 is also composed of a planar component (for example, half of the diazachrysene of NSC 329398) and a second cationic substituent (for example, one of the tertiary amine substituents of NSC 328398) (Figure 4c). Furthermore, zone 3 is a hydrophobic moiety (for example, one of the ACQ components of Q2-15) linked to the cationic substituent of zone 2 (Figure 4c). At this time, the linker between zones 2 and 3 is a flexible alkyl bridge, as observed in previously identified ACQ-based SMNPIs.[29,31]

With regard to the occupation of three pharmacophore zones, increasing SMNPI size is an important consideration with regard to drugability. On the one hand, the increase in size will increase potency via new inhibitor–enzyme contacts and greater occupancy of the BoNT/A LC substrate binding cleft. On the other hand, increasing the size of SMNPIs by the incorporation of new components must be closely monitored with respect to bioavailability issues. For example, it is widely accepted that lipophilicity (Log*P*) is the an important property for druglikeness[44–46] regardless of the target, and that compounds with Log*P* values of less than five constitute the majority of orally available drugs,[44–46] and therefore serve as a benchmark for drug development. Hence, maintaining druglike physical properties in SMNPIs, such as Log*P* values less than five, and a general balance between polar and nonpolar properties, will be critically important during the optimization of SMNPIs guided by the 3-zone pharmacophore.

Identification of a novel inhibitor scaffold that occupies the three zones of the refined pharmacophore

To further validate the 3-zone pharmacophore hypothesis, we sought to database mine novel BoNT/A LC inhibitor scaffolds possessing the additional hydrophobic center (either aromatic or aliphatic) corresponding to zone 3 occupancy. Thus, in the 3D search query, a new hydrophobic center was constrained

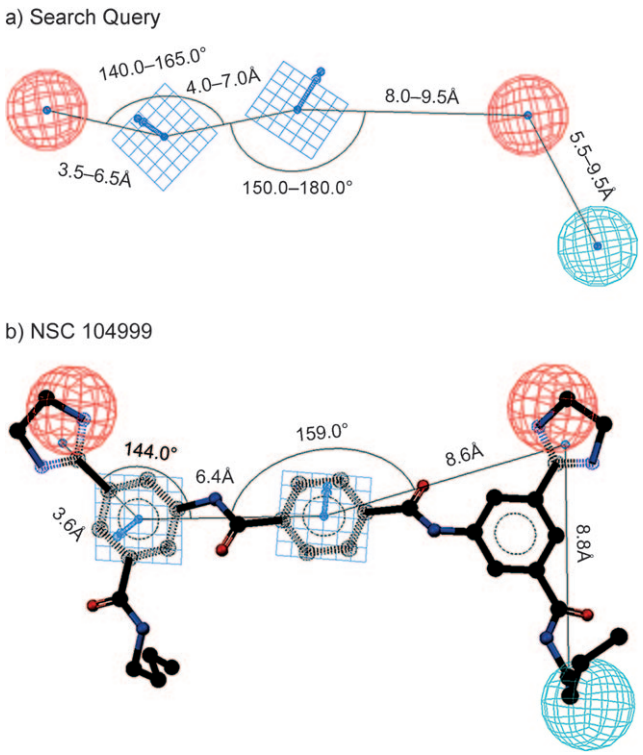


Figure 6. The BoNT/A LC search query incorporating a hydrophobic component based on the refined three zone pharmacophore: a) The search query: the hydrophobic component is shown as a cyan mesh sphere; all other components and colors are as indicated in Figure 2; b) The search query mapped to NSC 104999; all atom colors are as indicated in Figure 2.

within a distance range of 7.5 Å (±2.0 Å) from the cationic center of the query fitting within zone 2 (Figure 6a), corresponding to the distance between the centroid of the zone 3 quinoline of Q2-15 and the superimposed zone 2 cationic substituents shown in Figure 4c. Finally, the distance range between the two aromatic components of the search query was relaxed to reflect the length of the NSC 328398 diazachrysene.

A second round of 3D database mining with the new search query (using the NCI Open Repository as the database) resulted in ten hits, two of which were available for ordering and testing in our in vitro HPLC-based assay.[28,29,38–42] Of the two, NSC 104999 (Figure 7) provided 42% inhibition (at 20 μM concentration; data shown graphically in figure 2a of the Supporting Information), and possesses an IC_{50} value of 16.5 μM ±2.3. To ensure that the SMNPI was not acting via Zn chelation, its activity was measured in the presence of 0, 5, 10, 25, and 50 μM Zn concentrations, and its percent BoNT/A LC inhibition remained constant (data shown in figure 2b of the Supporting Information). The structurally novel SMNPI mapped to the search query is displayed in Figure 6b. Moreover, the compound is predicted to possess a Log*P* value of 4.4,[47] which is within the range of orally available drugs (that is, less than five[44–46]). Whereas NSC 104999 possesses a molecular mass of 650.77 Da, which is slightly higher than many reported orally available drugs, it should be noted that there are drugs (for example, HIV protease inhibitors) of high molecular mass (that is, >500 Da) that are orally available.[45]

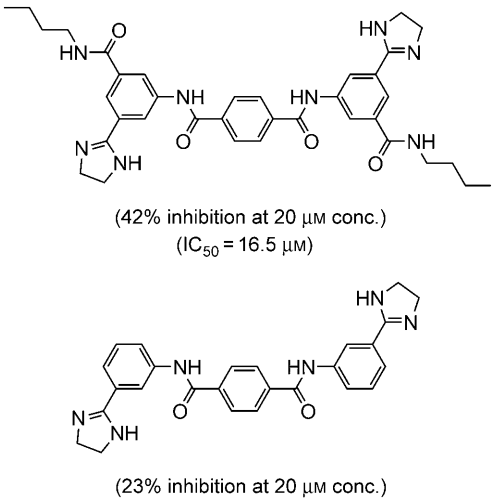


Figure 7. Two-dimensional structures of three-zone inhibitor NSC 104999 and analogue NSC 35839, a compound that can only occupy two zones of the pharmacophore. The weaker inhibitory activity of NSC 35839 supports our hypothesis that occupation of all three pharmacophore zones increases potency. For NSC 104999, aromatic rings have been labeled to facilitate further structural description in the Results and Discussion.

Alignment of NSC 104999, NSC 328398, and Q2-15 in the 3-zone pharmacophore is displayed in Figure 8. Based on this model, it is clear that the two cationic imidazoline substituents

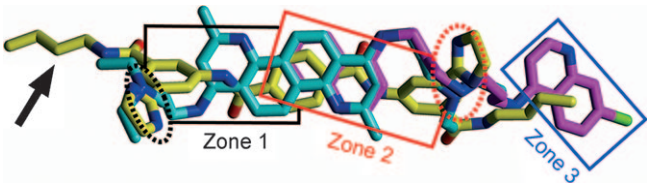


Figure 8. Novel SMNPI NSC 104999 superimposed on NSC 328398 and Q2-15. Atom colors are as indicated in Figure 4, with NSC 104999 carbon atoms colored yellow. The black arrow indicates the possibility of a new hydrophobic contact.

of NSC 104999 occupy the same 3D space as the tertiary amines of NSC 328398. Moreover, a portion of the butylamide substituent on NSC 104999 ring C (see Figure 7 for phenyl ring lettering) partially occupies the 3D space canvassed by the Q2-15 ACQ corresponding to pharmacophore zone 3 (Figure 8). Interestingly, the terephthalamide core of NSC 104999 is a radical departure from previously identified inhibitors,[28,29,31] being composed of only phenyl rings (versus heterocyclic aromatics[28,31]) bridged by amide bonds. Nevertheless, Figure 8 shows that the steric occupancy of this inhibitor fits within the 3-zone pharmacophore. Finally, the second butylamide substituent (indicated by the arrow in Figure 8) of NSC 104999 seems to provide evidence for another undefined hydrophobic area flanking zone 1 of the pharmacophore; the corresponding ethyl substituent on the zone 1 tertiary amine of 328398 appears to reinforce this possibility. Future SAR explorations will delineate the details of this potential new contact site.

To further examine our hypothesis that NSC 104999 occupancy of zone 3 increases its inhibitory potency, a congener, NSC 35839 (Figure 7), which does not possess butylamide substituents, and therefore can only occupy zones 1 and 2 of the pharmacophore, was examined for activity. Consistent with this limitation, this compound provided only 23% BoNT/A LC inhibition (at 20 μM concentration; data shown graphically in figure 2a of the Supporting Information), which, under the same assay conditions, is approximately half of the activity provided by NSC 104999.

Finally, although occupancy of zone 3 of the refined pharmacophore provides an additional opportunity to achieve optimal potency, NSC 104999 as a lead that occupies all three pharmacophore zones, is less potent than NSC 240898, which occupies only zones 1 and 2 (Figure 4c). A likely explanation is that NSC 240898 possesses superior binding site complementarity in zones 1 and 2 compared to NSC 104999. For example, the N–H component of the aromatic indole affords an additional hydrogen bonding interaction with a probable acceptor. This is lacking in the analogous aromatic component of NSC 104999. However, in light of our unsuccessful attempts to optimize NSC 240898 within zones 1 and 2 (data not shown), we believe that the incorporation of a structural component occupying zone 3 will provide new opportunities to optimize the potency of this SMNPI.

Conclusions

As evidenced by the discovery of four new inhibitors (NSCs 104999, 375162, 377363, and 328398), two of which are structurally novel (that is, NSC 104999 and NSC 328398), our gas-phase pharmacophore model is an independent paradigm for identifying unique BoNT/A LC SMNPI chemotypes. Moreover, as the model is continually being improved by the inclusion of new data, its ability to guide the identification of novel SMNPIs is also improved. In turn, incorporation of such new SMNPIs into the model results in new rounds of refinement. This process is iterative, with the discovery of every new SMNPI serving to increase the resolution of our understanding of the chemical and steric properties necessary for small molecule mediated BoNT/A LC inhibition. Even without reliable models of enzyme binding, this information will afford the synthetic optimization of the SMNPI scaffolds.

Experimental Section

Computational

All molecular modeling studies were performed on a Dell Precision 690 workstation running Linux Red Hat Enterprise version 4. SMNPI superimpositions and alignments were performed using Insight II (version 2005) software (Accelrys, San Diego, CA), and energy refinement was carried out using the Discover program (Accelrys) (cff91 force field) as a module within Insight II. Search query generation, compound library database mining, and SMNPI conformer generation were performed using Catalyst (version 4.11) software (Accelrys). In Catalyst, conformer ensembles for each SMNPI were generated using the 'Best' quality method, with an energy thresh-

old of 20 kcal mol^{-1} from the global minima, and a 250 conformer limit.[48] Database mining was performed using the Best Flexible Search Databases/Spreadsheet method. Figures 2 and 4 were generated using Catalyst (version 4.11) and Figures 3 and 5 were generated using Insight II (version 2005).

Formation of 3D search queries

Two similar, active lead compounds, NSC 240898 and NSC 341909, were used to generate the initial 3D search query (that is, the two zone hypothesis). For each molecule, the "function mapping" utility in Catalyst was used to locate two cationic centers and two ring arene centers. Location constraints were placed on each center, and the four centers were merged into a hypothesis. The three distances and two angles shown in Figure 2 were measured for each molecule, and distance and angle constraints that encompassed both molecules were placed in one query. The location constraints were deleted and the remaining search query was saved. To generate the search query for three zone compounds, an additional hydrophobic center was added to the previous search query. Subsequently, to make the query internally consistent, the distance and angle constraints were relaxed to fit NSC 328398 and Q2-15, thereby allowing for more compounds to be located.

In vitro testing

The HPLC-based assay used to quantify BoNT/A LC metalloprotease activity has been described in previous publications.[39–43,49] In brief, the assay utilizes an N-terminal acetylated, C-terminal aminated, synthetic peptide identical in sequence to residues 187–203 of SNAP-25. Compounds with intrinsic fluorescence quenching capability do not interfere with the activity measurements of this assay because substrate hydrolysis is determined by HPLC separation of the products from the substrate, followed by measurement of the peak areas. Assay mixtures consist of 40 mM HEPES-0.05% Tween (pH 7.3), BoNT/A recombinant LC, peptide substrate, 0.5 mg mL^{-1} bovine serum albumin, and various concentrations of SMNPIs. Assays are run at 37 °C, quenched by the addition of TFA, and analyzed by reverse-phase HPLC. To eliminate compounds that are Zn chelators, the SMNPIs are assayed in the presence of excess Zn^{II} salts. Percent inhibition measurements were performed in triplicate, and in all cases standard deviations were less than ±25%. IC_{50} values were determined by measuring BoNT/A LC activity at nine different SMNPI concentrations and in the absence of the SMNPI. The SMNPI concentrations in these measurements were determined by estimating the IC_{50} value and moving in one log increments in either direction of the estimated value. The resulting kinetic data were then fit to the Langmuir isotherm (1) using nonlinear regression analysis.

$$V_i/V_o = 1/1 + ([I]/IC_{50})^h \quad (1)$$

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