Computer-Aided Identification, Synthesis, and Biological Evaluation of Novel inhibitors for Botulinum Neurotoxin Serotype A

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ABSTRACT

Botulinum neurotoxins (BoNTs) are among the most potent biological toxins known to humans, and are classified as Category A bioterrorism agents by the Centers for Disease Control and prevention (CDC). There are seven known BoNT serotypes (A-G) which have been thus far identified in literature. BoNTs have been shown to block neurotransmitter release by cleaving proteins of the soluble NSF attachment protein receptor (SNARE) complex. Disruption of the SNARE complex precludes motor neuron failure which ultimately results in flaccid paralysis in humans and animals. Currently, there are no effective therapeutic treatments against the neurotoxin light chain (LC) after translocation into the cytosols of motor neurons. In this work, high-throughput in silico screening was employed to screen a library of commercially available compounds from ZINC database against BoNT/A-LC. Among the hit compounds from the in-silico screening, two lead compounds were identified and found to have potent inhibitory activity against BoNT/A-LC in vitro, as well as in Neuro-2a cells. A few analogues of the lead compounds were synthesized and their potency examined. One of these analogues showed an enhanced activity than the lead compounds.

Keywords: Botulinum neurotoxin BoNT/A-LC inhibitor SNAPtide SNAP-25 HTP in silico screening

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1. Introduction

Botulinum neurotoxins (BoNTs) produced by anaerobic bacteria *Clostridium botulinum* are the most potent biological toxin known, and they are classified as Category A bioterrorism agents by the Centers for Disease Control and Prevention (CDC). Although the toxin has found its way to the applications of medical treatments and cosmetic improvements, the potential use as a bio-weapon in terrorism still largely remains. The BoNT/A serotype has lethal dose of 1.3 ng/kg, and it is estimated that one gram of the aerosolized toxin can kill as much as 1 million people. Therefore it is a necessity to develop the therapeutic countermeasures against the BoNTs.

All serotypes function by the same mechanism, but target different SNARE proteins (SNAP-25, syntaxin or synaptobrevin) and sequences. Through experimental evidence BoNTs are known to vary in structure substantially among the serotypes, and as such, substrates also vary substantially. Currently there are no known inhibitors providing broad-spectrum inhibition against BoNTs. Thus, most of the literature has focused on the development of inhibitors against BoNT/A; the most potent toxin and the major cause of human botulism.

There are seven serologically distinct toxin types designated A to G. Type A is the most potent toxin, followed by types B and F toxin. Types A, B and E are commonly associated with systemic botulism in humans. The toxin is a two-chain polypeptide with a 100 kDa heavy chain joined by a disulfide bond to a 50 kDa light chain. This light chain is a zinc metalloprotease that cleaves the SNARE protein complex at a neuromuscular junction. As a result, the release of acetylcholine at the neuromuscular junction is ceased and leads to flaccid paralysis and eventual respiratory failure.

2. Results and Discussion

2.1. High-throughput virtual screening

The initial attempts at designing catalytic inhibitors of BoNT/A was focused on peptidomimetics. The tetrapeptide RRGC was found to have strong inhibitory activity (K<sub>i</sub> = 157 nM) against BoNT/A-LC. The X-ray co-crystal structure of RRGC in BoNT/A-LC (PDB ID: 3C88) provides us the key feature of the interaction of the inhibitor with the protein.

In the attempt to identify new BoNT/A-LC inhibitors, a large-scale virtual screening of over one million commercially available compounds was performed and resulted in the purchase of compounds for subsequent experimental evaluations. Traditionally many screening programs such as DOCK employ a simple two-term scoring function consisting of intermolecular van der Waals and electrostatic terms to rank-order compatibility of ligands with a target. A relative new scoring function developed by the Rizzo laboratory, termed “molecular footprint similarity (FPS)” score, utilizes the standard scoring function as a decomposition of the energy by per-residue contributions.

High-throughput virtual screen utilizing FPS score was conducted on BoNT/A-LC (PDB: 3C88, 1.60 Å resolution) using tetrapeptide RRGC [K<sub>i</sub> = 157 nM] as the reference ligand. The rational was to find small molecule inhibitors that possessed similar activity to the RRGC tetrapeptide while providing more robust scaffolds for chemical modification. As the result of the virtual screening, 99 compounds were ultimately selected, purchased, and assayed in vitro.

2.2. High-throughput biological screening

A high-throughput SNAPtide assay was used to screen the 99 selected compounds from the virtual screening. The assay carried out with protein concentration of 100 nM, the addition of 0.15 mM ZnCl<sub>2</sub> and 1.25 mM DTT. The quantity of SNAPtide was 5 μM, and the results are shown in Table 1.

Based on the IC<sub>50</sub> values thus obtained from the assay, compounds with FPS<sub>VDW+ES</sub> scores of greater than 0.90 appear to have better activity, and the structures and the footprint overlay of the best 2 hit compounds with RRGC are shown in Figure 1.

2.3. Cell-based Assay

Based on the SNAPtide screening results, two of the best hit compounds ChemDiv 5762-1843 and ChemDiv E843-1064 were selected for cell-based assay as lead compounds. For this assay, two lead compounds were examined for their activities in Neuro-2a cells inoculated with BoNT/A-LC. Results are shown in Figure 2 and Figure 3. ChemDiv 5762-1843 and ChemDiv E843-1064 exhibited graded inhibition at 10 μM. Specifically, 10 μM of each compound inhibited the quantity of cleaved SNAP25 after BoNT/A-LC was transfected into the cells. These two lead compounds were not cytotoxic to Neuro-2a cells at the concentration used.

<table>
<thead>
<tr>
<th>Zinc ID/ChemDiv ID</th>
<th>ChemDiv&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DCE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FPS&lt;sub&gt;VDW+ES&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>FPS&lt;sub&gt;VDW&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>FPS&lt;sub&gt;ES&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Ν&lt;sup&gt;f&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRGC</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3</td>
<td>31±14</td>
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<td>0.37</td>
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<td>ZINC00305216</td>
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<td>ZINC03662371</td>
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<td>0.93</td>
<td>0.56</td>
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<td>ZINC04894271</td>
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<td>3</td>
<td>157±5</td>
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<td>ZINC12112516</td>
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<td>0.92</td>
<td>0.41</td>
<td>0.51</td>
<td>3</td>
<td>159±16</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Zinc database ID number. <sup>b</sup>ChemDiv ID number. <sup>c</sup>DCE scores in kcal/mol. <sup>d</sup>FPS score in units of normalized Euclidian distance where 0 represents the best overlap. FPS<sub>VDW</sub> and FPS<sub>ES</sub> scores range from (0,2) and FPS<sub>VDW+ES</sub> range from (0,4). <sup>f</sup>Number of times the assay was run.
Figure 1. 2D chemical structure and 3D docked structures of hit compounds (A) ChemDiv ID 5762-1843 and (B) ChemDiv ID E843-1064. The VDW and ES footprint overlay of RRGC (red) and hit compounds (blue) (C) ChemDiv ID 5762-1843 and (D) ChemDiv ID E843-1064.

Figure 2. Western blot analysis of the inhibition of SNAP-25 cleavage by BoNT/A-LC in Neuro-2a cells by ChemDiv 5762-1843 (SB-BNI-01): 1) 10 μM, 2) 20 μM, 3) 50 μM, 4) 100 μM, 5) no inhibitor, 6) SNAP 25 marker.

Figure 3. Western blot analysis of the inhibition of SNAP-25 cleavage by BoNT/A-LC in Neuro-2a cells by ChemDiv E843-1064 (SB-BN-01): 1) no inhibitor, 2) 10 μM, 3) 20 μM, 4) 50 μM, 5) 100 μM, 6) SNAP 25 marker.
2.4. Synthesis of lead compounds and analogues

The retrosynthetic analysis of ChemDiv 5762-1843 led to two components (Scheme 1). The first component 3 was prepared starting from 4-fluoroaniline 1 in two steps. The other component 5 was prepared from imidazolidine-2,4-dione 4 with 2-fluorobenzyl bromide. Then, the Knoevenagel condensation of 3 with 5 using ethanolamine gave ChemDiv 5762-1843 (SB-BNI-01). Synthesis of ChemDiv E843-1064 was also based on the coupling of two components (Scheme 2). Quinazolinedione 9 was prepared from commercially available 6-nitropiprinol 6 in three steps. Oxadiazole 12 was synthesized from 4-trifluoromethyl-benzonitrile 10 in two steps. Coupling of 9 with 12 thus gave ChemDiv E843-1064 (SB-BN-01).

The resynthesized two hit compounds were fully characterized by \(^1\)H and \(^{13}\)C NMR and high resolution mass spectroscopy. In order to obtain a proof that the resynthesized compounds possess the same biological activities, SB-BNI-01 and SB-BN-01 were subjected to the SNAPtide assay \textit{in vitro}. As Table 2 shows, SB-BNI-01 and SB-BN-01 exhibited the same IC\(_{50}\) values as the ChemDiv compounds within error. Thus, the structures of the ChemDiv compounds were assured.

Following the same synthetic strategy, a few analogues of the hit compounds were prepared (Figure 4) and their IC\(_{50}\) values determined by SNAPtide assay (Table 2). SB-BNI-03 has \textit{para}-substitution at the center phenyl ring, but it did not drastically change the IC\(_{50}\) value. On the other hand, \textit{meta}-substitution at the center phenyl ring was detrimental to the inhibition activity. SB-BN-02 was unexpectedly obtained during an attempted synthesis of one-carbon shorter SB-BN-01 analogue, which resulted in double alkylation. It is worth mentioning that SB-BN-02 exhibited higher potency than SB-BN-01.

![Scheme 1](image1.png)

![Scheme 2](image2.png)

![Figure 4](image3.png)
3. Conclusion

In summary, two promising lead compounds ChemDiv 5762-1843 and ChemDiv E843-1064 were identified from a high-throughput virtual screening implementing FPS and screened against BoNT/A-LC. Both leads showed IC<sub>50</sub> values better than a reference inhibitor, RRGC. These lead compounds were then assayed for their activities in Neuro-2a cells against BoNT/A-LC, and found to be active at concentrations as low as 10 μM. Efficient synthetic routes to the lead compounds were established. The resynthesized compounds, SB-BNI-01 and SB-BN-01, were fully characterized and exhibited the same IC<sub>50</sub> values in the SNAPtide assay as the ChemDiv compounds. Three new analogues were synthesized and assayed, and one of them, SB-BN-02 exhibited better activity than the leads. Further studies on the SAR of lead compounds as well as structural biology of protein-inhibitor co-crystals are actively underway in these laboratories. A flow chart, summarizing the computer-aided drug discovery process described here, is shown in Figure 5.

![Flow chart for the computer-aided discovery of novel BoNT/A inhibitors](image)

**Figure 5.** Flow chart for the computer-aided discovery of novel BoNT/A inhibitors

4. Experimental Section

4.1. General Methods

Reaction solvents were reagents grade and freshly dried, degassed and distilled before use. Anhydrous N,N-dimethylformamide (DMF) and acetonitrile were purchased from Acros Organic and used without further purification. HPLC grade acetonitrile and water were purchased from EMD. Chemicals and reagents were purchased from Alfa Aesar or Sigma-Aldrich and used without further purification unless otherwise noted. <sup>1</sup>H NMR was measured on a Bruker Avance 400 MHz, Bruker Avance 300 MHz, Bruker Avance 400 MHz or Bruker Avance 500 MHz spectrometer at room temperature. <sup>13</sup>C NMR was measured on a Bruker Avance 400 MHz or Bruker Avance 500 MHz spectrometer at room temperature. Proton chemical shifts (d) are reported in parts per million (ppm) relative to deuterated solvents as the internal standard. Coupling constants (J) are reported in Hz. Analytical HPLC in reverse phase was carried out with a Shimadzu LC-2010AHT HPLC system using a Waters Nova-Pak C18 (60 Å, 4 μm, 3.9 mm X 150 mm) analytical column with water/acetonitrile as mobile phase. Melting points were measured on a Thomas-Hoover Capillary melting point apparatus and are uncorrected. TLC analyses were performed using Merck DC Alufolien 60F254 aluminum pre-coated silica gel plates. Flash column chromatography was carried out using Silicycle SiliaFlashP60® silica gel (particle size 40-63 μm). High-resolution mass spectrometric analyses were carried out by ICB&D at Stony Brook University. Unless otherwise noted all reactions were carried out under nitrogen atmosphere in oven-dried glassware using standard Schlenck techniques.

### 4.2. Virtual Screening using FPS

A high-throughput virtual screening of over one million molecules from the ChemDiv subset of the ZINC database (http://zinc.docking.org) was conducted on New York Blue, an 18 rack IBM Blue Gene/L massively parallel supercomputer located at Brookhaven National Laboratory using DOCK version 6.5. Prior to docking, the most updated ChemDiv database was downloaded and presorted by rotatable bonds and split into 10 subsets of ~100,000 molecules using the DOCK database filter. Subsequently, energy grids for BoNT/A-LC (PDB: 3C88) were generated using the grid program. Then, each molecule was flexibly docked to the BONT/A-LC grid (DOCK FLX protocol) and the single lowest-energy pose was retained.

Following high-throughput virtual screening, the footprint-based rescoring methodology reported by Baliani et al. was implemented to enrich the library of docked molecules. First, the co-crystallized reference ligand RRGC was minimized on the BoNT/A-LC Cartesian coordinates within the binding pocket. This was implemented using both a hydrogen optimization followed by a weak restrained minimization (restraint of 10 kcal/mol). Following reference minimization, each molecule of the docked library was subsequently minimized in Cartesian space using the restrained minimization protocol. Last, electrostatic, van der Waals, and hydrogen bond footprint similarity scores were computed using normalized Euclidian distance for each molecule docked versus the reference using DOCK 6.5.

Subsets 1 through 5 and subsets 6 through 10 each contained ~500,000 molecules were rank-ordered by the DOCK Cartesian energy score (DCE). The top 45,000 of each combined subsets (total ~90,000 molecules) were then clustered using MACCS fingerprints as implemented in the MOE<sup>®</sup> program with the Tanimoto coefficient of 0.75. The resulting cluster heads were further rank-ordered by: 1) standard DOCK score (DCE<sub>VDW+ES</sub>), 2) van der Waals footprint similarity score (FPS<sub>VDW</sub>), 3) electrostatic footprint similarity score (FPS<sub>ES</sub>), 4) H-bond footprint similarity score (FPS<sub>HB</sub>), 5) sum of van der Waals and electrostatic footprint similarity score (FPS<sub>VDW+ES</sub>). The top 250 molecules rank-

### Table 2. IC<sub>50</sub> values of resynthesized lead compounds and their analogs in the SNAPtide assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB-BNI-01 (ChemDiv 5762-1843)</td>
<td>24 ±2 (22 ± 2*)</td>
</tr>
<tr>
<td>SB-BNI-02</td>
<td>&gt;50</td>
</tr>
<tr>
<td>SB-BNI-03</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>SB-BN-01 (ChemDiv E843-1064)</td>
<td>27 ± 4 (24 ± 3*)</td>
</tr>
<tr>
<td>SB-BN-02</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

*IC<sub>50</sub> value for the ChemDiv compound
ordered by each criteria were then plotted and examined by visual inspection and consistency to the reference footprint. Based on this approach, 99 compounds were selected and purchased for biological testing against BoN-LC.

4.3. Biology

4.3.1. SNAPtide Assay

**Materials:** BoNT/A-LC truncated 1–425 was provided by Dr. Swaminathan at Brookhaven National Laboratory, Upton, NY. RRGC was provided by Dr. Swaminathan at Brookhaven National Laboratory, Upton, NY. Corning 96-well black microplates (Corning, NY). SNAPtide (List, Biological Laboratories, Inc., Campbell, CA). Full length BoNT/A (Metabiologics, Inc., Madison, WI). Primary antibody, anti-SNAP-25 mouse monoclonal IgG (200 μg/mL; Santa Cruz Biotechnology, Dallas, TX). Secondary antibody, donkey anti-mouse HRP conjugated (200 μg/mL; Santa Cruz Biotechnology).

The activity of BoNT/A-LC was measured in 96 well plates by the use of a Synergy 2 plate reader at 485 nm excitation and 528 nm emission. Stock solutions of inhibitors were prepared at 10 mM with DMSO. Assays contained 40 mM Heps (pH 7.4) buffer, 100 nM of enzyme, 0.15 mM ZnCl₂, 125 mM DTT, and various concentrations of inhibitor in a final volume of 100 μL. Assay was initiated by the addition of 5 μM SNAPtide. Initial rates were measured from the linear region of each assay, 100 to 300 s. IC₅₀ values were determined by the equation IC₅₀ = ([I]Vᵣ/[I-Vᵣ]), where [I] is the concentration of inhibitor Vᵣ is the initial rate in the absence of inhibitor, and V is the initial rate in the presence of inhibitor.

4.3.2. Cell-based Assay

Inhibitors were assessed for their ability to protect SNAP-25 from cleavage by BoNT/A as described with slight modifications. Briefly, Neuro-2a cells (ATCC CCL-131) were grown on 75 cm² tissue culture flasks in Eagle’s Minimum Essential Medium with salt containing 2 mM l-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1% penicillin-streptomycin, 1.0 mM sodium pyruvate and 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO₂ and 95% air. Once cells were 70% confluent they were seeded in culture flasks in Eagle’s Minimum Medium and cells were grown for an additional 24 hours. After 48 hours, the medium was replaced with serum-free medium and cells were grown for an additional 48 hours. Cells were then incubated with 1 μg of full length BoNT/A and various concentrations of inhibitors for 48 hours. Cells were harvested and proteins were separated by SDS-PAGE using a 12.5% gel. Proteins were transferred onto a nitrocellulose membrane that was blocked with milk followed by treatment with 1:1000 dilution of SNAP-25 mouse monoclonal antibody and subsequent treatment with 1:2000 dilution of donkey anti-mouse HRP. The membrane was treated with West Dura Chemiluminescent Substrate (Pierce) and exposed to x-ray film.

4.4. Chemistry

4.4.1. 3-(2-Fluorobenzyl)imidazolidine-2,4-dione (5)

To the solution of hydantoin 4 (1.00 g, 1.00 mmol) and 2-fluorobenzyl bromide (1.794 g, 9.95 mmol) in MeCN (40 mL) was added K₂CO₃ (2.762 g, 2.00 mmol) and the reaction was heated to 80 °C for overnight. The reaction was diluted with DCM (40 mL), filtered through Celite and concentrated. The crude was purified by recrystallization from DCM/hexanes to give 5 as white solid (1.724 g, 87%): m.p.: 133-134 °C; ¹H NMR (300 MHz, Chloroform-d): δ 4.03 (s, 2H), 4.78 (s, 2H), 5.89 (bs, 1H), 7.04-7.14 (m, 2H), 7.27-7.38 (m, 2H); ¹³C NMR (100 MHz, Chloroform-d) δ 35.92 (J = 4.6 Hz), 46.51, 115.62 (J = 21.2 Hz), 122.59 (J = 14.6 Hz), 124.23 (J = 3.7 Hz), 129.74 (J = 8.1 Hz), 130.17 (J = 3.6 Hz), 157.96, 160.59 (J = 246.7 Hz), 170.81; HRMS (ESI-TOF) m/e calcd for [M+H⁺]⁺ C₅H₄F₂N₂O₂: 209.0726, found 209.0729 (Δ 1.4 ppm).

4.3.2. N-(4-Fluorophenyl)-2-chloroacetamide (2)

To the solution of 4-fluoronicotine (0.600 g, 5.40 mmol) and TEA (0.600 g, 5.94 mmol) in DCM (15 mL) was added dropwise chloroacetyl chloride (0.671 g, 5.94 mmol) and the reaction was stirred at room temperature for overnight. The reaction was washed with water (20 mL), brine (20 mL), dried over MgSO₄ and concentrated to give 2 as off-white solid (1.01 g, crude quantitative): m.p.: 131-132 °C; ¹H NMR (300 MHz, Chloroform-d): δ 4.21 (s, 2H), 7.08 (t, J = 8.60 Hz, 2H), 7.53 (dd, J = 8.60°4.71 Hz, 2H), 8.23 (bs, 1H). The analytical data was consistent with literature data.¹⁹

4.4.3. General procedure for the synthesis of 2-(formylphenyloxy)acetamide (3)

To the solution of hydroxylaldehyde (2.0 mmol) and 2 (2.0 mmol) in MeCN (20 mL) was added K₂CO₃ (3.0 mmol) and the reaction was heated to 80 °C for overnight. The reaction was cooled to room temperature, diluted with DCM (50 mL), filtered through Celite and concentrated. The crude was purified by column chromatography (silica gel, EtOAc/Hexanes = 5% → 30%) to afford 3a-c.

4.4.4. N-(4-Fluorophenyl)-2-(2-formylphenyloxy)-acetamide (3a)

White solid, 77%; m.p.: 123-125 °C; ¹H NMR (300 MHz, Chloroform-d): δ 4.71 (s, 2H), 6.99-7.11 (m, 3H), 7.27 (t, J = 7.62 Hz, 1H), 7.65 (m, 1H), 7.79-7.88 (m, 3H), 10.05 (bs, 1H), 10.11 (s, 1H); ¹³C NMR (100 MHz, Chloroform-d) δ 67.72, 113.52, 115.66 (J = 22.4 Hz), 121.31 (J = 7.8 Hz), 122.28, 125.04, 133.96 (J = 2.8 Hz), 136.32, 136.38, 157.08, 159.47 (J = 241.8 Hz), 165.50, 191.32; HRMS (ESI-TOF) m/e calcd for [M+N⁺]+ C₂₅H₁₉F₂N₂O₂: 396.0699, found 396.0722 (Δ 7.7 ppm).

4.4.5. N-(4-Fluorophenyl)-2-(3-formylphenyloxy)-acetamide (3b)

White solid, 70%; m.p.: 101-102 °C; ¹H NMR (300 MHz, Chloroform-d): δ 4.68 (s, 2H), 7.06 (t, J = 8.49 Hz, 2H), 7.28 (d, J = 8.01 Hz, 1H), 7.50-7.60 (m, 5H), 8.29 (bs, 1H), 10.05 (s, 1H); ¹³C NMR (100 MHz, Chloroform-d) δ 67.61, 113.87, 115.86 (J = 22.6 Hz), 121.28, 122.04 (J = 7.8 Hz), 124.97, 130.70, 132.60 (J = 2.9 Hz), 138.14, 167.49, 159.80 (J = 243.1 Hz), 165.43, 191.46; HRMS (ESI-TOF) m/e calcd for [M⁺Na⁺]+ C₂₅H₁₉F₂N₂O₂Na⁺: 398.0929, found 398.0966 (Δ 4.1 ppm).

4.4.6. N-(4-Fluorophenyl)-2-(4-formylphenyloxy)-acetamide (3c)

White solid, 72%; m.p.: 118-120 °C; ¹H NMR (300 MHz, Chloroform-d): δ 4.71 (s, 2H), 7.04-7.15 (m, 4H), 7.55-7.59 (m, 2H), 7.92 (d, J = 8.55 Hz, 2H), 8.22 (bs, 1H), 9.95 (s, 1H); ¹³C NMR (100 MHz, Chloroform-d) δ 67.43, 115.13, 115.88 (J
4.4.7. General Procedure for the synthesis of SB-BNI compounds

To the suspension of 5 (1.0 mmol) and 3 (1.0 mmol) in water (5 mL) was added EtOH (3 mL) and ethanolamine (3.0 mmol), and the reaction was heated to 105 °C for overnight. The reaction mixture was cooled to room temperature and diluted with water (20 mL). The product was collected by vacuum filtration and dried over phosphorus pentoxide under vacuum. The product was purified by recrystallization in MeOH to afford BNI compound.

4.4.8. (Z)-2-(2-((1-(2-Fluorobenzyl)-2,5-dioxoimidazolidin-4-ylidene)methyl)phenoxy)-N-(4-fluorophenyl)acetamide (ChemDiv57621843, SB-BNI-01)

White solid 61%: m.p.: 222-224 °C; ¹H NMR (400 MHz, DMSO-d₆): δ 4.74 (s, 2H), 4.88 (s, 2H), 6.85 (s, 1H), 7.02 (m, 2H), 7.14-7.40 (m, 7H), 7.62 (m, 3H), 10.34 (s, 1H), 10.77 (s, 1H), 13.16; ¹³C NMR (100 MHz, DMSO-d₆) δ 35.81, 35.85, 67.06, 105.83, 112.41, 115.78, 115.97, 116.00, 121.82, 121.90, 122.22, 123.53, 123.67, 125.03, 125.07, 127.28, 130.05, 130.09, 130.17, 130.82, 130.88, 135.08, 135.11, 154.75, 155.96, 157.57, 159.10, 159.96, 161.54, 164.23, 167.17; HRMS (ESI-TOF) m/z calcd for [M+H]⁺ C₂₅H₂₁F₂N₂O₂: 464.1416, found 464.1422 (Δ 1.3 ppm).

4.3.9. (Z)-2-(3-((1-(2-Fluorobenzyl)-2,5-dioxoimidazolidin-4-ylidene)(methyl)phenoxy)-N-(4-fluorophenyl)acetamide (SB-BNI-02)

White solid, 64%: m.p.: 212-213 °C; ¹H NMR (400 MHz, DMSO-d₆): δ 4.73 (s, 2H), 4.78 (s, 2H), 6.56 (s, 1H), 7.05 (m, 1H), 7.14-7.38 (m, 9H), 7.67 (m, 2H), 10.11 (s, 1H), 10.96 (s, 1H); ¹³C NMR (100 MHz, DMSO-d₆) δ 35.89, 35.93, 67.64, 110.25, 115.60, 115.67, 115.76, 115.90, 115.97, 116.16, 122.11, 122.19, 123.27, 124.43, 125.59, 125.04, 125.07, 130.05, 130.09, 130.19, 130.37, 134.54, 135.14, 135.16, 155.28, 157.57, 158.46, 159.10, 159.96, 161.54, 164.50, 166.92; HRMS (ESI-TOF) m/z calcd for [M+H]⁺ C₂₅H₂₁F₂N₂O₂: 464.1416, found 464.1436 (Δ 4.3 ppm).

4.3.10. (Z)-2-(4-((1-(2-Fluorobenzyl)-2,5-dioxoimidazolidin-4-ylidene)(methyl)phenoxy)-N-(4-fluorophenyl)acetamide (SB-BNI-03)

Yellow solid 62%: m.p.: >240 °C; ¹H NMR (400 MHz, DMSO-d₆): δ 4.73 (s, 2H), 4.76 (s, 2H), 6.56 (s, 1H), 7.05 (d, J = 8.76 Hz, 2H), 7.15-7.38 (m, 6H), 6.75 (m, 2H), 10.18 (s, 1H), 10.78 (s, 1H); ¹³C NMR (100 MHz, DMSO-d₆) δ 35.79, 35.84, 67.51, 110.66, 115.53, 115.70, 115.76, 115.92, 115.96, 122.01, 122.09, 123.54, 123.69, 125.03, 125.06, 125.29, 126.35, 129.98, 130.02, 130.07, 130.15, 131.73, 135.15, 135.17, 158.57, 158.63, 159.08, 159.95, 161.52, 164.37, 166.72; HRMS (ESI-TOF) m/z calcd for [M+H]⁺ C₂₅H₂₂F₂N₂O₂: 464.1416, found 464.1454 (Δ 8.2 ppm).

4.3.11. 5-Chloroethyl-3-(4-fluoromethylphenyl)-1,2,4-oxia-diazole (12)

A solution of chloropropionyl chloride (0.031 g, 0.25 mmol) in benzene (0.35 mL) was added drop wise to of 4-trifluoromethyl-N-hydroxybenzamidine (0.1 g, 0.49 mmol) in benzene (3 mL). The reaction mixture was refluxed at 80 °C for 12 hours. The reaction as monitored by TLC. The reaction mixture was evaporated under vacuum to obtain the crude product, which was purified by flash column chromatography using silica gel and ethyl acetate/hexanes (gradient 5-10 % ethyl acetate/hexanes) as eluent to give 12 as colorless oil (52 mg, 79 % yield); ¹H NMR (500 MHz, Chloroform-d): δ 3.46 (t, J = 6.9 Hz, 2H), 4.00 (t, J = 6.9 Hz, 2H), 7.75 (d, J = 8.1
Hz, 2H), 8.21 (d, J = 8.1 Hz, 2H); $^{13}$C NMR (125 MHz, Chloroform-d) δ 30.54, 39.57, 123.95 (q, J = 271.0 Hz), 126.11 (q, J = 3.6 Hz), 128.06, 130.19, 133.24 (q, J = 32.5 Hz), 167.74, 177.03; HRMS (ESI-TOF) m/e calcd for [M+H]$^+$ $C_9$H$_7$ClF$_3$N$_2$O$_6$: 277.0350, found 277.0348 (Δ 0.7 ppm).

4.3.17. E843-1064 (SB-BN-01)

A mixture of 6,7-methylenedioxy-quinazoline-2,4(1H,3H)-dione (50 mg, 0.23 mmol), 5-chloroethyl-3-(4-trifluoromethylphenyl)-1,2,4)oxadiazole (72 mg, 0.26 mmol) and potassium carbonate (36 mg, 0.26 mmol) in dimethyl sulfoxide (2 mL) was heated to 60 °C for 6 h. The crude reaction mixture was diluted with ethyl acetate and washed with water several times. The organic layer was dried over magnesium sulfate, filtered and evaporated to give the crude mixture which was purified by column chromatography (silica gel, EtOAc/Hexanes = 30% → 60%) to afford SB-BN-01 as white solid (37 mg, 35 % yield), m.p.: 224-226 °C; $^1$H NMR (300 MHz, Acetone-d$_6$): δ 3.42 (t, J = 6.9 Hz, 2H), 4.48 (t, J = 6.9 Hz, 2H), 6.15 (s, 2H), 6.77 (s, 1H), 7.31 (s, 1H), 7.91 (d, J = 8.1 Hz, 2H), 8.24 (d, J = 8.1 Hz, 2H), 10.25 (bs, 1H); $^{13}$C NMR (100 MHz, Acetone-d$_6$) δ 24.73, 37.32, 95.14, 102.59, 104.76, 107.73, 129.75, 126.00, 127.81, 131.80, 136.86, 144.42, 150.02, 153.94,161.40,167.05, 178.42; HRMS (ESI-TOF) m/e calcd for [M+H]$^+$ $C_{30}$H$_{18}$F$_3$N$_6$O$_6$: 447.0916, found 447.0938 (Δ 4.9 ppm).

4.3.20. SB-BN-02

A mixture of 6,7-methylenedioxy-quinazoline-2,4(1H,3H)-dione (50 mg, 0.24 mmol), 5-chloroethyl-3-(4-trifluoromethyl-phenyl)-1,2,4)oxadiazole (70 mg, 0.27 mmol) and potassium carbonate (37 mg, 0.27 mmol) in dimethyl sulfoxide (3 mL) was heated to 60 °C for 6 h. The crude reaction mixture was diluted with ethyl acetate and washed with water several times. The organic layer was dried over magnesium sulfate, filtered and evaporated to give the crude mixture which was purified by column chromatography (silica gel, EtOAc/Hexanes = 1% → 30%) to afford SB-BN-02 as white solid (24 mg, 15 % yield), m.p.: 196-198 °C; $^1$H NMR (300 MHz, Acetone-d$_6$): δ 5.65 (s, 2H), 5.90 (s, 2H), 6.23 (s, 2H), 7.30 (s, 1H), 7.55 (s, 1H), 7.88 (m, 4H), 8.21 (d, J = 8.0 Hz, 4H); $^{13}$C NMR (100 MHz, Acetone-d$_6$) δ 37.44, 40.44, 95.68, 103.26, 105.72, 108.85, 122.66, 125.36, 125.95, 125.99, 126.03, 126.07, 126.11, 126.15, 127.91, 127.95, 130.13, 130.32, 132.19, 132.32, 132.52, 132.64, 137.50, 145.06, 150.46, 155.05, 160.01, 167.30, 167.37, 175.94, 176.45; HRMS (ESI-TOF) m/e calcd for [M+H]$^+$ $C_{22}$H$_{17}$F$_3$N$_6$O$_7$: 659.1108, found 659.1108 (Δ 0 ppm).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/xxxxxxxxxxxxxxxxxxxxxxx

References and Notes

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Yu-Han Gary Teng, William T. Berger, Natasha M. Nesbitt, Kunal Kumar, Trent E. Balius, Robert C. Rizzo, Peter J. Tonge, Iwao Ojima* and Subramanyam Swaminathan
Institute of Chemical Biology and Drug Discovery, Department of Chemistry, and Department of Applied Mathematics and Statistics, Stony Brook University; Biological, Environmental & Climate Sciences Department, Brookhaven National Laboratory, New York, United States

HTP in-silico screening of ZINC database
SNAPtide assay in vitro
Inhibition of SNAP 25 cleavage in Neuro-2a cells
Two promising lead compounds and their analogues