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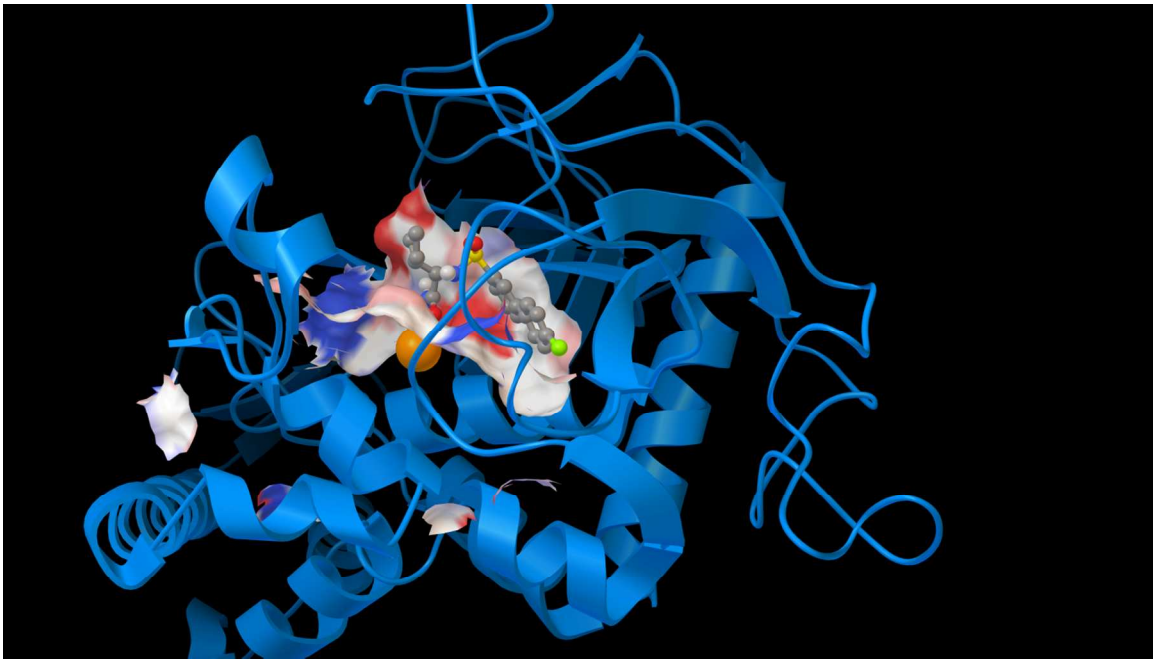

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Introduction

The botulinum neurotoxin (BoNT) is the toxic agent that causes botulism, an illness that results in muscle paralysis and in severe cases, death.[1] The neurotoxin is produced by the bacteria, *Clostridium botulinum* and is the most lethal biological toxin known to man with an LD_{50} of 1.3 ng/kg of body weight for the BoNT/A serotype.[2] It is estimated that one gram of the neurotoxin can kill 1 million people.[1] Due to its potency and ease of production there is a concern that the toxin could be used as a weapon for bioterrorism, making it necessary for the development of therapeutic countermeasures.[3, 4]

The BoNT is composed of heavy (~100 kDa) and light chains (~50 kDa). The heavy chain binds to nerve cells and inserts the light chain into the cytosol.[5] The light chain (LC) is a zinc metalloprotease that is responsible for proteolysis of soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein.[6] Cleavage of a SNARE protein causes termination of neurotransmission and is irreversible.[1] There are seven different serotypes (A-G) of the neurotoxin, which all function by the same mechanism, but cleave different SNARE proteins and sequences.[5] The toxicity also varies with each serotype, with A being the most toxic to man followed by B and E.[2]

The only treatments currently available for BoNT intoxication are antibody based vaccines and physical therapy.[1, 4] Both of these treatment options are not practical for large infected populations and/or long delay between exposure and treatment. An alternative treatment are small molecule therapeutics that can specifically inhibit the LC metalloprotease both inside and outside the cell.[7] This would be an ideal treatment for the neurotoxin in a mass bioterrorism attack. Treatment of botulism due to the absorption of the bacterial would involve a combination of antibiotics to treat the bacteria infection and small molecule therapeutics to target the BoNT. There has been considerable effort in developing small molecule therapeutics,[8] however no small molecule has been approved for the treatment of BoNT/A intoxication.

Many different classes of molecules have been reported to inhibit the BoNT/A LC with IC_{50} values in the μM to nM range.[8-11] A number of inhibitors feature a "chemical warhead" such as a hydroxamic acid, which can coordinate to the zinc atom present in the catalytic active site.[12-15] The best hydroxamic acid inhibitors for the BoNT/A LC have reported IC_{50} or K_i values in 300-20 nM region. Generally, hydroxamic acids are a poor choice for small molecule inhibitors, due to the promiscuous binding nature of the hydroxamic acid for a variety of metals (Fe, Mg, Cu and Co) in the body.[16] However, suberoylanilide hydroxamic acid[17] has been approved for the treatment of lymphoma, which demonstrates the potential for hydroxamic acids as viable therapeutic options. Other reported BoNT/A LC inhibitors have been based on natural products (K_i = 6.7 μM)[18, 19] and quinolinol[20-22] derived molecules (K_i values reported in the 1.84-0.8 μM range).

In our efforts to improve on BoNT/A LC inhibitors we included a sulfonamide group in our chemical scaffold. The sulfonamide is prevalent in many therapeutic agents such as antibiotics[23], anti-diabetic[24], diuretics[25], and anticonvulsants[26]. Sulfonamides have also been incorporated in carbonic anhydrase[27, 28] and MMP inhibitors[29, 30]. Several inhibitors containing this group have also been reported for the BoNT/A LC with a k_i value of 6.3 μM for the best inhibitor.[14, 31] The sulfonamide is an ideal group for therapeutic drugs due to its ability to participate in hydrogen bonding, unique molecular geometry related to

molecular shape and excellent chemicophysical properties such as enhanced water-solubility and bioavailability.[29, 32] Currently this group has not been thoroughly explored as an inhibitor for the BoNT LC and could provide new chemical scaffolds that are therapeutically sustainable.

In this study we report the discovery of a sulfonamide hydroxamic acid inhibitor for the BoNT/A LC (IC_{50} = 10.09 ± 2.59 μM). Through a structure activity relationship (SAR) study of the parent compound we identified essential elements for inhibitor activity. A 2nd generation compound was synthesized based on critical structural elements and resulted in a 10-fold increase in inhibitor activity.

Results and Discussion

Chemistry

The synthesis of a 320 member sulfonamide hydroxamic acid library consisted of a four step synthetic route on solid phase support as depicted in Scheme 1. The structural diversity of the library was incorporated with various Fmoc-protected amino acids and a library of sulfonyl chlorides.

The synthesis begins with coupling the Fmoc-protected amino acid to the hydroxylamine resin following standard peptide coupling procedures with *N*,*N*'-diisopropylcarbodiimide (DIC) and 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) in DMF.[12] The Fmoc group was selectively removed from the coupled amino acid by treating the resin with 25% pipiperidine (PIP) in DMF. The sulfonamide bond was prepared by reacting the free amine with sulfonyl chloride and 2,6-lutidine in DCM using a microwave reactor. The sulfonamide hydroxamic acid was cleaved from the resin with 50% trifluoroacetic acid (TFA) in DCM to give the final product (Scheme 1). The compounds were purified by prep HPLC.

Inhibitor Assay

The library was screened for BoNT/A LC inhibitors via a FRET based assay using commercially available substrate and LC.[31] Compounds discovered from the inhibitor screen were validated as BoNT/A LC inhibitors and the IC_{50} value determined. The inhibitor screen resulted in the identification of **1** as an inhibitor for the BoNT/A LC (Fig. 1). Compound **1** is composed of a hydroxamic acid (R_1) with isoleucine (R_2), linked via a sulfonamide bond to a biphenyl (R_3) with a chloride attached (R_4).

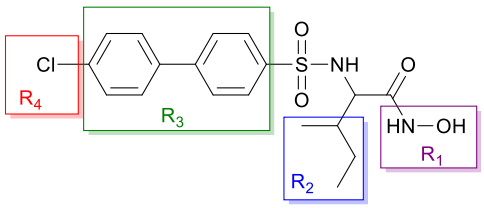
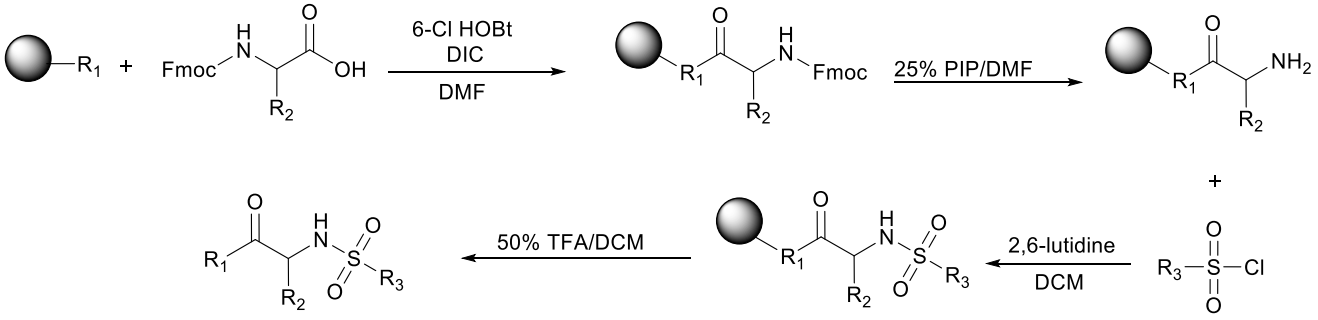


Fig. 1 Compound **1** (IC_{50} = 10.09 ± 2.59 μM) with highlighted regions for SAR study.

Based on the initial inhibitor structure, we devised a SAR study (Fig. 1) to identify important structural elements required for BoNT/A LC inhibition.

It is known that the hydroxamic acid is a zinc binding group and is featured on many competitive inhibitors for the BoNT/A LC.[13] We believe that the hydroxamic acid is important for our inhibitor scaffold and will participate in a competitive mode of inhibition. We tested this hypothesis by replacing the hydroxamic acid with an amide (**2**) that does not chelate zinc and carboxylic acid (**3**), which has a low affinity for zinc binding (Table 1). Both **2** and **3** displayed no inhibition for the BoNT/A LC. This evidence supports a competitive mode of inhibition through direct chelation of the zinc atom with the hydroxamic acid group of our scaffold.

The amino acid side chain of **1** is a sec-butyl group and we believe that a hydrophobic side chain is a good match for the hydrophobic nature of the BoNT/A LC active site[33]. The amino acid and side chain were explored with other hydrophobic groups to improve binding in the active site (Table 2). Incorporating proline (**4**) represented a very different side chain resulting in a different molecular shape from **1** and did not show any inhibition for the BoNT/A LC. In an effort to maximize hydrophobic interactions, phenylalanine was inserted into the scaffold (**5**), but did not show any inhibitor activity. These results lead us to examine other branched alkyl chains similar to the parent sec-butyl group. Valine (**6**) and leucine (**7**) were incorporated into the scaffold and resulted in IC_{50} values ~3 and ~2 folds (**6** and **7** respectively) lower in activity relative to **1**. The alkyl branching appears to be important for binding and the active site is sensitive to small changes in the alkyl branching. The isopropyl and isobutyl groups of



Scheme 1. Synthesis of the sulfonamide hydroxamic acid scaffold

Table 1. Variation of the R_1 group, with their corresponding IC_{50} values. (>100 implies no significant inhibition)

Compound	R_1 Group	IC_{50} (μM)
1	NHOH	10.09 ± 2.59
2	NH_2	>100
3	OH	>100

6 and **7** respectively, provided a suitable range of alkyl branching to explore in our scaffold. Also due to the large increase in the IC_{50} values for **6** and **7** we hypothesize that other alkyl groups, such as *tert*-butyl would not improve binding. Therefore the sec-butyl group is the optimal side chain for our scaffold.

By incorporating an amino acid into our inhibitor scaffold we include a stereocenter at the amino acid α carbon. In addition, isoleucine contains an additional stereocenter at the β carbon. To fully evaluate the amino acid side chain the stereochemistry of our scaffold was also evaluated for inhibitor activity. We believe stereochemistry will be important for binding, due to the selectivity observed with the alkyl branching and stereochemistry has been previous reported to affect BoNT/A

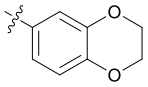
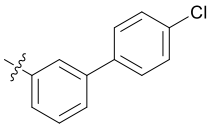
Table 2. Various amino acids (R_2) incorporated into the parent scaffold with corresponding IC_{50} values. (>100 implies no significant inhibition)

Compound	R_2 Group	IC_{50} (μM)
4	L-Proline	>100
5	L-Phenylalanine	>100
6	L-Valine	38.16 ± 5.90
7	L-Leucine	24.76 ± 6.49
8	D-Leucine	25.87 ± 7.62
9	D-Isoleucine	6.91 ± 1.12
10	L-allo-Isoleucine	41.79 ±12.41
11	D-allo-Isoleucine	11.85 ± 2.73

inhibitor activity[34]. There was no significant difference in activity for either stereoisomers of leucine (**7** and **8**). Incorporating the D-isoleucine (**9**) into the scaffold caused a slightly more active inhibitor relative to the parent. However, the opposite enantiomer at the β-methyl position of isoleucine (**10**), displayed a 4-fold decrease in inhibitor activity relative to **1**. Following this trend, the stereochemistry of both the α and β carbons were inverted (**11**) relative to **1**, resulting in a slightly higher IC_{50} value compared to **1**. These results confirm that the branched alkyl side chain plays a crucial role in binding to the BoNT/A LC. Based on the sensitivity of the stereochemistry at the β-carbon we are confident that the sec-butyl group is the optimal alkyl branching group for our scaffold. Also the orientation of the biphenyl ring in the active site is significant for inhibitor activity and D-isoleucine is preferred in our scaffold for binding in the BoNT/A LC.

Based on the hydrophobic nature of the active site[14, 35] we explored different aromatic groups in the R_3 region. (Table 3). No significant inhibition was observed when the biphenyl ring was replaced with a phenyl group (**12**). This is a key result, which demonstrations the importance of the biphenyl ring for our class of inhibitors. As expected other ring systems (**13**) also did not show improved activity. Adjusting the substitution pattern of the biphenyl ring (**14**) did result in a slightly improved inhibitor relative to **1** (Table 3). These results are consistent with a hydrophobic active site that can accommodate large hydrophobic molecular structures, such as the adamantane group, which is contained in one the most potent BoNT/A LC inhibitors (K_i = 27 nM).[35]

Table 3. Variation of the R_3 group with their corresponding IC_{50} values. (>100 implies no significant inhibition)

Compound	R_3 Group	IC_{50} (μM)
12		>100
13		20.75 ± 3.32
14		8.14 ± 1.79

With the significance of the biphenyl ring established the substituent on the ring was explored for inhibitor activity (Table 4). Replacing the chlorine with hydrogen (**15**) resulted in a 2-fold decrease in inhibitor activity relative to **1**. Indicating that the chloride is participating in binding with the BoNT/A LC active site. Replacing chlorine with larger hydrogen bonding groups such as methoxy (**16**) or acetylamide (**17**)

Table 4. Variation of the R_4 group with their corresponding IC_{50} values. (>100 implies no significant inhibition)

Compound	R_4 Group	IC50 (μM)
15	H	27.69 ± 9.86
16	OCH_3	>100
17	$NHCOCH_3$	>100
18	F	9.60 ± 1.07
19	CH_3	>100

displayed no inhibitor activity. The larger groups were used to probe for intermolecular hydrogen bonding interactions in the active site. However due to the largely hydrophobic active site with few residues available for hydrogen bonding they did not improve binding and caused more steric hindrance. As expected, replacement of the chlorine with fluorine (**18**) gave no difference in inhibitor activity relative to **1**. Substitution of the chlorine for methyl (**19**) resulted in no inhibition of the BoNT/A LC. This is an interesting result since methyl is a known bioisostere for chloride, due to the similar size of both groups. This observation indicates the halide is involved in intermolecular interactions with residue(s) in the active site that cannot occur with the methyl group. Based on the SAR data for the chloride, there appears to be a polar interaction between the chlorine and the active site. These halide-residue interactions have been previously observed in X-ray crystallography structures of small molecules bound in the BoNT/A LC active site.[33]

Based on the results of the SAR study we synthesized a 2nd generation compound containing structural elements that were deemed essential for inhibitor activity. 2nd generation compound **20** is composed of the hydroxamic acid, D-isoluecine and the `4-chloro-biphenyl-3-sulfonamide (Fig. 2). The IC_{50} value for **20** is 0.95 μM which is 10-fold more active than **1**.

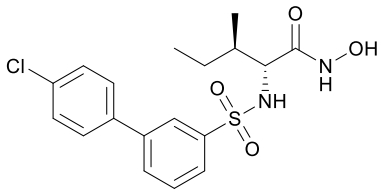


Fig. 2 2nd generation inhibitor (**20**) with an IC_{50} of 0.95 ± 0.60 μM for the BoNT/A LC.

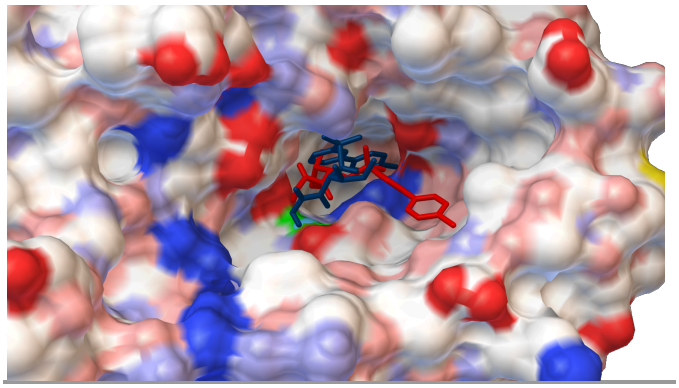


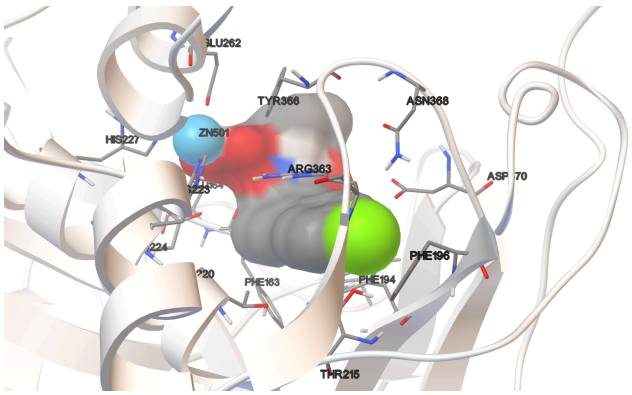
Figure 3. Left: Computational docking models of **1** (red) and **20** (blue) in the BoNT/A LC active site (PDB # 4EJ5). Right: Docking model of **20** in the active site, displaying residues in close proximity to **20** (color scheme for **20;** grey = carbon; red = oxygen; blue = nitrogen; green = chlorine, yellow = sulfur).

Molecular Docking

In addition to the experimental data, **1** and **20** were computationally docked[36] into the active site of the BoNT/A LC (PDB # 4EJ5) to provide a model of inhibitor binding (Fig. 3). Based on the docking studies, **1** and **20** are observed to chelate the zinc atom in the active site. This observation is in agreement with experimental data supporting zinc chelation (Table 1).

The amino acid side chains of **1** and **20** are located on opposite sides of the active site. The amino acid side chain of **20** is positioned toward the opening of the active site cavity, while the side chain of **1** is pointed inside the active site cavity. It is unclear from the model how the BoNT/A LC active site is sensitive to changes in the alkyl branching.

The biphenyl rings of **1** and **20** are also orientated into different regions of the active site. The opposite stereochemistry of **1** and **20** cause both the side chains and biphenyl rings to occupy different regions in the active site. The biphenyl ring of **1** is located on the edge of the active site along the wall. Even though the biphenyl ring of **1** is in a solvent exposed region, it is in close proximity to aromatic residues (Phe369, Tyr251, Tyr366) that form π-π interactions resulting in moderate ligand binding affinity, which is observed experimentally. The biphenyl ring of **20** is located deep inside the active site cavity against the 370 loop (Fig. 3). In the cavity the biphenyl ring is in close proximity to Phe194, Phe196, Phe163 and the chloride is adjacent to Arg363. This particular arginine residue has been observed by X-ray crystallography to form polar intermolecular interactions with halides from other BoNT/A LC small molecule inhibitors.[14,33] We hypothesize that there is an interaction between the chloride and Arg363, which is important for binding, due to the lack of polar intermolecular interactions available in the active site. This hypothesis is supported by experimental data, which exhibited a decrease in inhibitor activity when the chloride was replaced with different groups.



The reported flexibility[31, 33] of the active site pocket, could explain why we do not observe the Arg363 chloride interaction, but our model does indicate that the chloride is in proximity to this residue.

Conclusion

From a library of sulfonamide hydroxamic acids we discovered a competitive inhibitor for the BoNT/A LC. Based on a logical SAR study we explored the structural elements required for small molecule binding to the active site. This study directed our strategy for a 2nd generation compound that had a 10-fold lower IC_{50} value relative to our lead compound. Molecular docking supported our experimental data and provided a binding model for our 2nd generation compound.

Future SAR studies will focus on the substituents located on the biphenyl ring, specifically studying electron withdrawing groups and their substitution pattern. These SAR studies will completely optimize this region and aid in further lowering the IC_{50} values of future small molecule inhibitors.

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Electronic Supplementary Information (ESI) available: [Experimental procedure, Assay protocol and conditions details]. See DOI: 10.1039/b000000x/

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