

8. RATIONAL DESIGN OF BOTULINUS NEUROTOXIN THERAPIES

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BOTULINUS NEUROTOXIN INHIBITORS

Short Term Goal: Discover novel inhibitors of Botulinus neurotoxin A

Long Term Goal: Design oral inhibitors of Botulinus neurotoxin A

BACKGROUND

Botulinus neurotoxin A (Botox A) is one of several protein toxins from *Clostridium botulinum*, which cause paralytic syndromes resulting from the blockage of neurotransmitter release. These toxins are all zinc endopeptidases acting in the neuronal cytosol: Botox B, D, F and G as well as tetanus toxin attack specifically VAMP (also called synaptobrevin) – a protein of synaptic vesicles; Botox A and E cleave SNAP-25 and Botox C acts on syntaxin – both proteins of the presynaptic membrane

Botox A is a 150K protein made up of a 50K light chain (amino acids 1-448) and a 100K heavy chain (amino acids 449-1296) which are held together by a disulfide bond (C430-C454). The heavy chain contains a transmembrane domain (residues 659-681) which inserts into the neuronal cell membrane and enables the light chain to access the interior of the neuron. Intracellular reduction of the interchain disulfide activates the proteolytic activity of the light chain (zinc-binding domain HELIH, residues 223-227) which then cleaves the protein SNAP-25 and disables the docking mechanism required for exocytosis of the neurotransmitter.

SNAP-25 is one component of the so-called SNARE complex, which is responsible for docking synaptic vesicles and fusion to the cell membrane as the immediate precursor event to transmitter release. The SNARE complex is a four-helix bundle made up of two small proteins, namely VAMP and syntaxin, and a larger protein SNAP-25 which doubles back on itself and provides two of the four threads of the helix bundle. SNAP-25 also contains a lipid anchor region (amino acids 85-92) between the two helical threads, which lines up with transmembrane domains at the C-termini of both VAMP and syntaxin (Poirier et al, Nature Structural Biology 5:765,1998). Whereas Botox A selectively cleaves the Gln187-Arg203 bond near the C-terminus of SNAP-25, the other botulinus neurotoxins each selectively cleave a different peptide bond within one of the three target proteins which comprise the SNARE complex.

A repeating motif exists within the sequences of all three of the proteins of the SNARE complex which, when introduced in the form of synthetic peptides of about ten residues in length, inhibits the actions of botulinus toxins on the SNARE complex (Rossetto et al, Nature 372: 416, 1994). For example one version of the repeating motif present in VAMP, designated V2 and having the sequence 62ELDDRADALQ71, blocks the neurotoxic actions of Botox A and B when the peptide is injected into cultured Aplysia neurons. The significance of the repeating SNARE motif, which appears twice on each of the four threads of the SNARE helix bundle upstream of the cleavage sites, is not well understood. Presumably it acts as a recognition site for binding of some other biomolecule(s), and may also be used by Botox as a binding-recognition element. In agreement with this, cross recognition of the target proteins by the various toxins occurs: Botox A inhibits VAMP proteolysis by Botox B, and Botox B and tetanus inhibit the cleavage of SNAP-25 by Botox A. Moreover serum albumin, which contains SNARE motifs within helical regions of its secondary structure (183DELDRD187 and 255DDRAD259), inhibits the cleavage of synthetic substrate by Botox A.

Thus although the mechanism of action of V2 (and other variants of the SNARE motif) is not proven, it could involve binding to a complementary recognition site on Botox resulting in inhibition of productive binding of Botox to the SNARE complex. Interestingly, possible complementary SNARE motifs (592KKVVK596 and 701KRNEK705 in the heavy chain, and 335KLFKFDK340 and 359KVLNRK364 in the light chain) exist in Botox A.

STRATEGY

Several possible strategies for inhibiting Botox exist:

- Block the transmembrane domain of Botox and prevent cell entry, e.g. complementary peptides, antibodies;
- Block the proteolytic site of Botox, e.g. substrate inhibitors, selective chelation of zinc;
- Block the interaction of Botox with binding-recognition motif, e.g. mimics of SNARE motif, which bind to complementary site(s) on Botox.

The first strategy has potential application prior to exposure to toxin (e.g., immunization), as well as for the deactivation of toxin in body fluids after exposure. The latter strategies, on the other hand, have the potential for providing total body treatment after toxin exposure - assuming that the therapeutic agent is able to cross cell membranes and inactivate toxin which has already entered the cell. Whereas agents based on active site inhibitors will have to be tailored to individual variants of Botox, therapies based on the SNARE motif have the potential to treat poisoning by all forms of Botox as well as tetanus.

Active sites of zinc proteases

The most widely studied of all the zinc proteases is carboxypeptidase A (CPA), and information on the mechanism of action of this enzyme serves a useful basis for understanding other related zinc enzymes such as Botox.

Detailed X-ray studies on CPA have illustrated that the tetradentate zinc atom sequesters a single water molecule and is tethered by coordination to the imidazole groups of His-69 and His-196 as well as to the carboxylate of Glu-270. CPA undergoes a conformational change on binding of the substrate Gly-Tyr, in which the guanidinium group of Arg-145 moves 2Å to form a salt bridge with the C-terminus of the substrate, the carboxyl group of Glu-270 moves 2Å away from the zinc atom and forms a salt bridge with the amino group of the substrate (can only occur with dipeptide), and the phenolic group of Tyr-248 moves 12Å to within 3Å of the scissile bond (Reeke et al, PNAS 57, 2220, 1967). Chemical modification studies have suggested that Tyr-248 is essential for the peptidase activity, but not the esterase activity, of CPA. A catalytic mechanism has been proposed in which the susceptible carbonyl oxygen of the substrate coordinates to zinc and the Glu-270 carboxylate attacks the carbonyl carbon to form a mixed anhydride intermediate, which is subsequently hydrolyzed by a base catalyzed mechanism involving the nascent water molecule (Zn-OH).

The best inhibitors of CPA are generally compounds that coordinate with the active site zinc and either sequester the zinc atom away from the enzyme or prevent access of the substrate to the catalytic center. These include thiols, amines/imines, flavonoids, and a range of anionic groups including carboxylates, phosphonates, etc. The most potent inhibitors will be those having an exact fit to the active site of the enzyme which is accompanied by strong attractive forces resulting in high affinity binding not only to the zinc atom but also to the enzyme's peptidic groups.

Due to its preference for basic amino acid residues, the enzyme most like Botox A is carboxypeptidase B. CPB differs from CPA in that it contains three disulfides (CPA has only one) and the coordination of zinc involves a free Cys thiol. Exchange of zinc for cadmium in CPB results in only esterase activity (no peptidase activity). Replacement of Asp-253 (which normally binds the basic sidechain of the substrate) with Lys gave a reversed-polarity mutant of human CPB which hydrolyzed hippuryl-L-glutamic acid (Edge et al, Protein Eng. 11, 1229, 1998).

The best inhibitors of CPA are benzyl- or alkyl-succinic acids and, assuming some parity between CPA and CPB, likely inhibitors of CPB would be aminoalkyl- or guanidinoalkyl-succinic acids. For reasons of specificity, it follows that those considerations which apply to CPB will likely also apply to Botox A. Accordingly, peptide libraries directed towards the active site of Botox A should be rich in basic and acidic moieties. Interestingly, acidic residues are also a characteristic of the SNARE motif, so that peptide libraries composed of these residues should interact with both the catalytic site and the SNARE motif site and act as dual inhibitors.

Regardless of mechanism, one approach to producing improved inhibitors of Botox is to identify the structural elements of V2 (and other SNARE motifs) that make it an effective inhibitor. Then reconstruct these components into a smaller and preferably nonpeptidic molecule, which would be able to traverse membranes, thereby providing not only access to the inside of cells, but also the potential for oral activity. A streamlined and efficient approach to this goal is to create a library of small semimimetic peptides containing the essential elements of the repeating motif in the SNARE complex, identify the most active peptide in the mixture by iterative deconvolution of the library, and then restructure the best semimimetic peptide identified into a fully fledged nonpeptide mimetic using computer molecular modeling techniques.

The important structural feature of the SNARE motif is comprised of an amino acid sequence made up of residues:

A-A-x-x-A-x-x

Where A = acidic residue and x = nonpolar or polar residue.

Due to the helical arrangement of these groups within the secondary structure of the SNARE proteins from which they are derived, the end result is a cluster of three neighboring negative charges juxtaposed by a nonpolar moiety. In other words, the required motif can be envisaged as three negatively charged groups, mounted in close proximity on a hydrophobic template. The simplest representation of this that comes to mind, is a benzene ring with three carboxymethyl groups attached at the 1, 3 and 5 positions. Other variations on this theme can also be easily

envisaged, and a number of commercially available compounds that fit this general scheme are being investigated for inhibitory activity in our laboratory, together with several peptide libraries which have been designed and synthesized with these considerations in mind.

DESIGN AND SYNTHESIS OF PEPTIDE LIBRARIES

One library has been synthesized which is designed to mimic the SNARE motif:



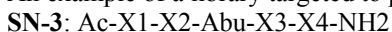
Where X1, X2, X3 & X4 are mixtures of Asp, Glu, Gln and Arg, and the LINKER group is 4-aminobutyric acid

The rationale for the design of the peptide minilibrary SN-1 is based on the preponderance of acidic residues in SNARE motifs, together with the occurrence of other residues in SNARE motifs which can provide solubility properties to the libraries – hence the inclusion of Arg and Gln in SN-1. The multiple negative charges represented by the Asp and Glu residues, when mounted around the flexible linker group 4-aminobutyric acid (Abu), should engender structural and conformational variations on the SNARE motif which will inhibit Botox.

Coincidentally, the Gln and Arg residues in SN-1 are also representative of the scissile bond (Q-R) in the Botox A substrate, so that the peptide minilibrary SN-1 doubles in a rudimentary way as a substrate mimic. This parallels the classic approach to inhibitor discovery, which is usually done by minor modification of substrate structure leading to compounds that bind to the active site but are not cleaved by the enzyme. Accordingly, incorporating residues of the scissile bond (Gln-Arg) into peptide libraries such as SN-1 should provide structural and conformational characteristics that might result in substrate-based inhibitors. Clearly SN-1 contains the potential for containing inhibitors based not only on the SNARE motif but also on substrate structure.

Another consideration for the design of peptide libraries with inhibitory potency is the inclusion of residues which would be expected to coordinate to the active site zinc atom of Botox, namely Cys and His as well as acidic residues. The approach here is to create a peptide library containing an array of zinc binding elements in a variety of different formats, wherein there should be individual peptides which recognize not only the zinc atom but also unique aspects of the active site of Botox A in which the zinc atom resides. The intent is to produce zinc targeted inhibitors that show selectivity for Botox over other zinc metalloenzymes.

An example of a library targeted to produce active site zinc inhibitors is:-



where Ac = acetyl, Abu = 4-aminobutyric acid, and X1, X2, X3, and X4 are mixtures of the amino acids Asp, Glu, His and Cys.

This library contains both active site zinc directed probes *and* variations on the SNARE motif within the same basis set (library) of peptides.

CONFORMATIONAL PROPERTIES OF TARGET BINDING SITES

Before intricate design work on inhibitors of Botox can be undertaken, details of the three-dimensional conformation of the target sequence must be elucidated. In the case of Botox A, the substrate SNAP-25 is believed to form part of a four-helix protein bundle that is anchored into the presynaptic membrane. Actually the cleavage site targeted by Botox A is near the C-terminus of SNAP-25, which is far removed from the central lipid anchor region of SNAP-25. Similarly the amino acid sequences representing the four SNARE motifs of SNAP-25 (S1, S2, S3 and S4) reside in the central part of the SNARE complex away from the lipid anchor domain found at one end of the tubular bundle. Accordingly, it is not clear if the environment surrounding the sequences representing not only the cleavage site but also the SNARE motifs is primarily polar (aqueous) or nonpolar (membrane lipid) in character.

As a precautionary measure we have conducted conformational calculations on these target sequences firstly in a nonpolar environment and subsequently in a polar environment. Semiempirical energy minimization calculations carried out on the substrate SNAP-25 (187-203) revealed that this peptide takes up a helical conformation in a nonpolar environment (in vacuo), but that the introduction of water molecules into the environment results in the helix unraveling. Accordingly, the conformation of this peptide is highly dependent on environment, although the spatial arrangement of amino acid sidechains in the critical region of the QR scissile peptide bond remains the same. It is not clear if Botox A is likely to preferentially recognize either the helical conformation or the disrupted conformation, and at present there is no way of knowing which of the two conformations would be appropriate for design work on potential active site inhibitors.

Likewise, semiempirical calculations on the SNARE motifs V2 and S3 have revealed helical structures in a nonpolar environment which become disrupted in the presence of water. Again, since there is no way of knowing which is the biologically relevant conformation, it is not possible to conduct design work based on the information presently available. Consequently we have elected to proceed with peptide library investigations in the hope that the identification of a bioactive semimimetic peptide will provide the answer to this question. Thus, eventually it should

be possible to identify from library screening, a potent inhibitory ligand containing the essential structural elements of the SNARE motif, which is sufficiently conformationally restricted that it can only take up one of the two SNARE motif conformations – either that found in membranes or that found in water. At that stage it will then be possible to use the appropriate conformational model for further detailed design work.

OPTIMIZATION OF ASSAY CONDITIONS

Several laboratories have reported assays of Botox A activity against various substrates. Rossetto et al (1994) investigated the action of Botox A against intact SNAP-25 and found that only the reduced form of the enzyme (pretreated with 10mM DTT for 30 min at 37C) could cleave the substrate. For the subsequent incubation with substrate, they used 5mM HEPES buffer pH 7.4 for 60 min at 37C, without the addition of supplementary zinc. These authors also reported that the enzyme was blocked by 10mM EDTA (zinc chelator) or by 1.4mM captopril (zinc endopeptidase inhibitor).

Schmidt & Bostian (1997) studied the activity of Botox A towards a number of short synthetic substrates derived from the cleavage site of SNAP-25 and found that peptides of at least 15 residues were required for cleavage by the reduced form of the enzyme. Incubations were carried out in 30mM HEPES buffer pH 7.3 in the presence of 5mM DTT, 250uM zinc and 1mg/ml BSA for 10 min. at 37C. Activity against these substrates required the presence of BSA and increased with increasing concentrations of BSA to a maximum effect at 1mg/ml BSA. The activity was blocked by 5mM EDTA or 100mM Tris (zinc chelators).

In this laboratory we have investigated the effects of pH and varying concentrations of zinc and DTT on the activity of the reduced enzyme. Reduction was accomplished by incubating the enzyme with 20mM fresh DTT at 37° for 30 min. in 50mM HEPES pH 8. After reduction optimal activity against a 17mer peptide from SNAP-25 (residues 187-203) was observed by preincubating the enzyme (5nM) in 15mM HEPES buffer pH 6.9 in the presence of 1uM zinc and 0.5mM DTT for 5 hr before adding the substrate (50uM). After 30 min. the reaction was terminated with an equal volume of 1% TFA and the products determined by HPLC. The activity was inhibited in the complete absence of zinc or in the presence of zinc concentrations above 10uM, and was also inhibited by 10mM DTT. These findings illustrate that Botox A, like many other zinc endopeptidases, requires the presence of zinc but is inhibited by excess zinc. In addition, since high concentrations of DTT can inhibit, possibly due to the zinc chelating properties of DTT, a careful balance between zinc and DTT concentrations must be engendered.

INHIBITORS OF BOTOX A

In our studies a number of compounds were found to inhibit the Botox A mediated cleavage of the 17-amino acid synthetic peptide substrate Ac-SNKTRIDQANQRATKML-NH₂, which derives from the C-terminal part (residues 187-203) of SNAP-25 and contains the scissile QR peptide bond targeted by Botox A (Table 1). Several thiol-containing compounds, namely dithiothreitol (widely used to reduce disulphide bonds in proteins), DMPS (a potent chelator of heavy metals) and Captopril (a clinical inhibitor of the zinc dipeptidase Angiotensin Converting Enzyme) block the cleavage of this substrate. However the non-thiol ACE inhibitor Lisinopril was ineffective, whereas the non-thiol prodrug ACE inhibitor enalapril was an effective inhibitor. The thiol compounds DMPS, captopril and DTT presumably act by a mechanism involving sequestration of the zinc atom at the active site of Botox A. The inhibitory activity of enalapril was unexpected but could suggest an affinity for the active site of Botox A which is not shared by lisinopril.

The synthetic peptide V2 was also able to inhibit Botox A, but at a higher concentration (5mM) than has been observed previously in vivo when SNAP-25 was the substrate (100uM, see Rossetto et al 1994). This may reflect the absence of a SNARE motif in the short synthetic substrate used for the present studies.

Interestingly serum albumin (10uM), which contains the SNARE motif in duplicate, inhibited cleavage of SNAP-25 (187-203) under our assay conditions which had been optimized for zinc (1uM) and DTT (0.5mM) concentrations. In contrast Schmidt & Bostian (J. Protein Chem. 16: 19, 1997) observed that in the presence of 250uM zinc and 5mM DTT cleavage of this substrate by Botox A only occurred in the presence of BSA. Our findings would suggest that this was probably due to sequestration of inhibitory levels of zinc/DTT by BSA.

Investigations of SN-1 on the Botox A cleavage of substrate has shown that this peptide minilibrary does inhibit the enzyme activity (Table 1). This suggests that variations on the SNARE motif present in the library mixture may interfere with binding of Botox to the substrate. However a series of peptides which were selected for their potential to represent simple variations on the SNARE motif, i.e. Glu-Glu, Glu-Glu-Leu, Glu-Glu-Glu and Glu-Pro-Glu-Thr, were generally inactive (data not shown), with the notable exception of Glu-Glu-Glu. A number of peptides with potential complementary sequences of the SNARE motif, i.e. Lys-Arg, Lys-Lys, Orn-Orn, Lys-Lys-Lys, Orn-Orn-Orn and Lys-Phe-Gly-Lys, were inactive as expected because the synthetic 17mer peptide substrate used for the assay lacks the repeating SNARE motifs that are present in the longer SNAP-25 natural substrate. However these

basic peptides would be expected to inhibit the cleavage of SNARE proteins by Botox enzymes in general, if they bind well to the SNARE recognition motifs in these proteins.

Interestingly, glycyrrhizic acid, which is a steroid glycoside containing 3 carboxylate groups, was able to inhibit Botox (Table 1). This finding, as with Glu-Glu-Glu, suggests that certain configurations of negative charges are able to approximate the SNARE motif and inhibit Botox A. However in the final analysis it would appear that the most potent inhibitor of Botox containing a SNARE motif variation is likely to be found in the peptide library SN-1 because each peptide in the mixture was present at 20uM concentration (Table 1).

Finally, the present experimental findings also indicate that the best inhibitors of Botox A are likely to be derived from thiol containing compounds (the inhibitory activity of DMPS is particularly remarkable). In agreement with this, the peptide minilibrary SN-3 turned out to be an exceptionally potent inhibitor of Botox A (Table 1). When the overall thiol concentrations of the inhibitors are compared (Table 1) it is apparent that the peptide library SN-3 is the most potent thiol inhibitor of Botox. Furthermore it is very likely that SN-3 contains a peptide(s) which is a more potent inhibitor than DMPS.

We are currently evaluating SN-3 inhibition of other related zinc protease neurotoxins namely Botulus B and tetanus neurotoxin. The intent is to deconvolute the library and determine which peptide in the mixture is the best inhibitor of Botox A and which peptide in the library is the best inhibitor of Botox B and tetanus. The expectation is that there will be sufficient differences in the active sites of the toxins to yield different optimal inhibitors from the same 'hinge' peptide minilibrary.

ACKNOWLEDGEMENTS

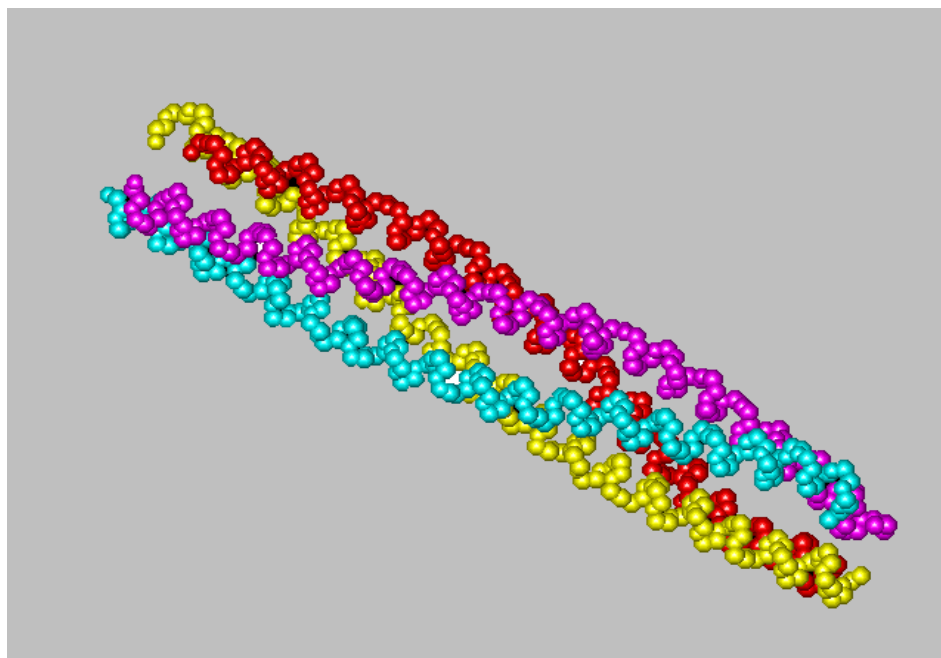
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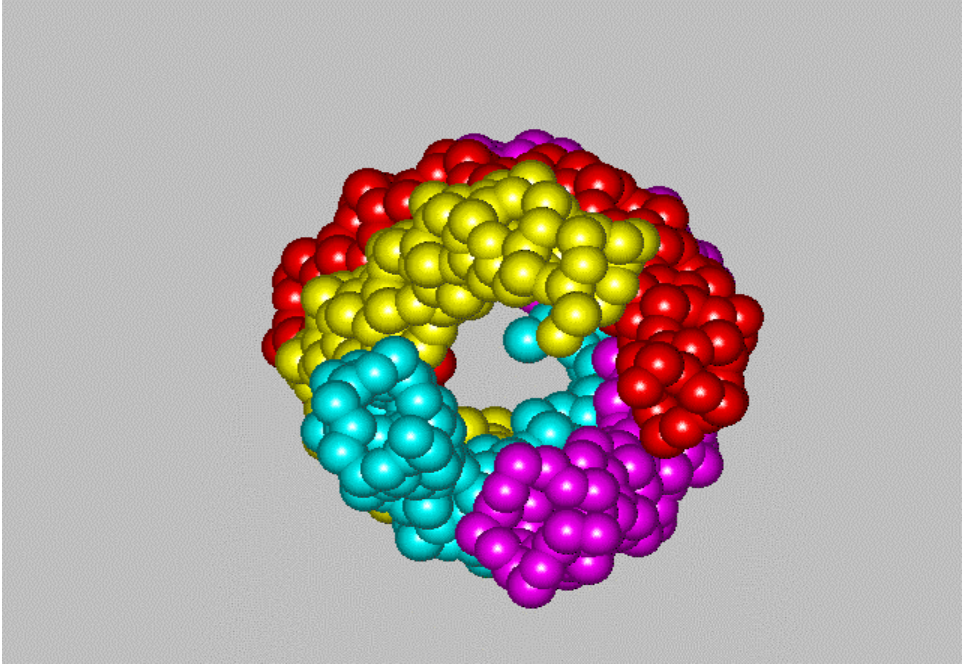
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FIGURES AND TABLES

SNARE Complex: Lateral



SNARE Complex: Top View



Details of SNARE complex, showing the substrate, V2 and S3

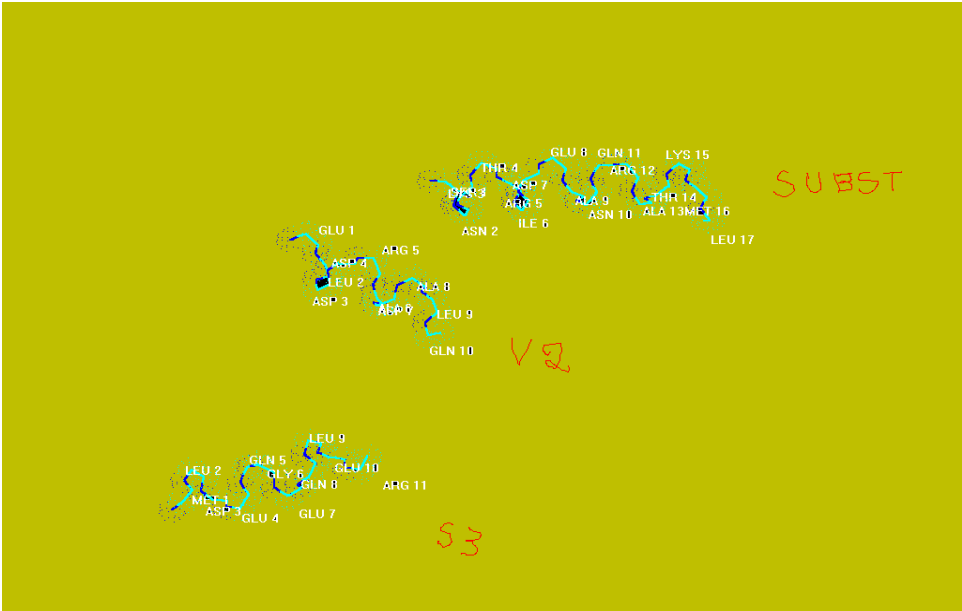


Table 1. Compounds found to inhibit the Botox A mediated cleavage.

Inhibitor	Concentration (mM)	% Inhibition*	-SH Concentration (mM)
Dithiothreitol	1	26	2
DMPS [‡]	0.1	72	0.2
Captopril	1	76	1
Lysinopril	5	7	0
Enalapril	5	40	0
V2 (ELDDRADALQ)	5	40	0
Glu-Glu-Glu	5	22	0
Glycyrrizic acid	5	55	0
Library SN-1	5 (20 μ M) [†]	16	0
Library SN-3	0.5 (2 μ M) [†]	51	0.125