DOI: 10.1002/anie.200705531

Medicinal Chemistry

The Strange Case of the Botulinum Neurotoxin: Using Chemistry and Biology to Modulate the Most Deadly Poison

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Keywords:
bioterrorism · botulinum neurotoxin · high-throughput screening · metalloenzymes · vaccine design

In the classic novella "The Strange Case of Dr. Jekyll and Mr. Hyde", Robert Louis Stevenson paints a stark picture of the duality of good and evil within a single man. Botulinum neurotoxin (BoNT), the most potent known toxin, possesses an analogous dichotomous nature: It shows a pronounced morbidity and mortality, but it is used with great effect in much lower doses in a wide range of clinical scenarios. Recently, tremendous strides have been made in the basic understanding of the structure and function of BoNT, which have translated into widespread efforts towards the discovery of biomacromolecules and small molecules that specifically modulate BoNT activity. Particular emphasis has been placed on the identification of inhibitors that can counteract BoNT exposure in the event of a bioterrorist attack. This Review summarizes the current advances in the development of therapeutics, including vaccines, peptides, and small-molecule inhibitors, for the prevention and treatment of botulism.

From the Contents

1. Introduction

Botulinum neurotoxins (BoNTs) are the most toxic poisons known to humans, with a lethal dose (LD_{50}) of approximately 1 ng per kg of body weight.[1] There are seven serologically distinct BoNTs (A–G); serotype A (BoNT/A) is the most potent serotype, possessing a toxicity 10^6-fold higher than cobra toxin and 10^{11}-fold greater than cyanide.[2] BoNTs are responsible for the potentially fatal disease botulism, commonly associated with food contamination as well as wound infection or colonizing infection in infants. BoNT intoxication is characterized by flaccid paralysis caused by the proteolytic cleavage of specific SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins critical for the release of the neurotransmitter acetylcholine from nerve cells. The lethality associated with botulism is due to advanced intoxication of the lung and heart, ultimately leading to either respiratory failure or cardiac arrest. Therefore, the fatal effects of the toxins are due not to target cell death, but rather to secondary events that depend on toxin-induced inhibition of acetylcholine release within the synaptic cleft.

The first clinical documentation of botulism comes from the publications of Justinus Kerner,[3,4] a German physician and poet who often referred to BoNT as the "sausage poison" because food poisoning was often associated with liver or blood sausages up to that period (it is of interest to note that *Botulus* is Latin for sausage). Kerner's initial report coincided with findings from Johann Georg Steinbuch. Both were small-town medical officers who, upon recommendation by the esteemed medical professor Johann Autenrieth, documented all the cases of food poisoning, which led to Autenrieth's publication in *Tübinger Papers for Natural Sciences and Pharmacology*.[5,6] However, it was Kerner that carried the study further, doing tests on animal subjects as well as his own body, and in fact postulated several therapeutic uses for BoNT.[4] The extent of his work prompted contemporaries at the time to nickname him "Wurst-Kerner." The bacterium responsible for botulism, *Clostridium botulinum*, was not identified until 1897, after microbiologist Emile Van Ermengem isolated the bacteria from ham that poisoned 13 Belgian musicians, 3 of whom died.[7]

The extreme potency and specificity of action of BoNTs make the toxin a very effective therapeutic agent and research tool.[8,9] Their utility covers a wide variety of disorders including those of a neuromuscular basis (for example, strabismus, blepharospasms, and hemifacial spasms) as well as non-neuromuscular disorders (for example, hyperhidrosis, myofascial pain, migraine headaches, and multiple sclerosis). Perhaps the most popular BoNT-associated application is cosmetic, where the commercial product Botox (based on BoNT/A) is used as an antiwrinkle agent.

Although BoNTs have many attractive characteristics, their potential use as a dangerous biological weapon remains. The extreme potency and lethality, ease of procurement and transport, and the need for prolonged intensive care among afflicted individuals make BoNTs a major terrorist and warfare threat.[10,11] Consequently, these biomacromolecules are classified as Category A agents by the US Centers for Disease Control and Prevention (CDC). Their use in biological warfare dates back to World War II, during which both Allied and Axis powers evaluated BoNTs as bioweapons. The Iraqi military has admitted that BoNTs were incorporated in specially engineered Scud missiles during the Gulf War. With the ongoing war on terror, there has become increased concern that terrorist groups will utilize BoNTs. Thus, substantial efforts have been made in the scientific community to obtain a clearer picture of BoNT

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action and to develop pharmaceuticals and vaccines for the treatment and prevention of botulism.

Since the vast majority of focus to date has been on BoNT/A and BoNT/B, with the former receiving the majority of attention, our discussion primarily involves these two serotypes, with particular emphasis on BoNT/A.

2. Molecular Mechanism of BoNT and Neurotransmitter Release

BoNTs are proteins with a molecular weight of about 150 kDa that are synthesized as single inactive polypeptide chains which become activated by intra- or extracellular proteolysis.[12] The active form of the toxin is a disulfide-linked dimer composed of a 100 kDa heavy chain (HC) coupled to a 50 kDa light-chain metalloprotease (LC). The BoNT serotypes share a high degree of homology, yet differ in their toxicity and molecular site of action. As shown in Figure 1, BoNT intoxication occurs through a multistep process involving each of the functional domains of the toxin, and can be described as the outcome of four discrete stages: 1) neurospecific binding at the neuromuscular junction, 2) internalization by endocytosis, 3) translocation and release of the LC into the cytosol, and 4) cleavage of the SNARE protein, thereby preventing vesicle fusion and acetylcholine release.[13,14]

2.1. Cell Binding and Internalization

BoNTs bind to cholinergic nerve terminals through their HC domains and are subsequently internalized by receptor-mediated endocytosis through a proposed double-receptor mechanism.[15] In such a scenario, the first step involves association of the toxin with the cell membrane and ganglioside to form a low-affinity complex. The second step involves lateral migration of the complex to a high-affinity protein binding site, which ultimately leads to endocytosis. The importance of ganglioside binding was demonstrated both in vitro and in vivo: BoNT/A was detoxified when incubated with an excess of gangliosides,[16] and knockout mice lacking gangliosides were less sensitive to BoNT/A and BoNT/B.[17] Additionally, mutational studies have shown that a single ganglioside and a single protein receptor comprise the receptor binding site for BoNT/B and BoNT/G.[18] Recent structural studies have confirmed the two-receptor model,[19] as evidenced by the detailed interaction between BoNT/B and both synaptotagmin and the G_{T1b} ganglioside.

It is generally presumed that the process of BoNT receptor-mediated endocytosis is very similar to that of most ligands internalized into cells.[14] However, it has been noted that a retrieval phase in the vesicle recycling mechanism may also provide a plausible route of entry.[20] Here, consideration has been given to the fact that nerves that undergo exocytosis have a vigorous and well-developed mechanism for membrane retrieval.[21] In the case of BoNT, research has inferred that endocytosis is mediated by recycling secretory vesicles. Recent findings have indicated

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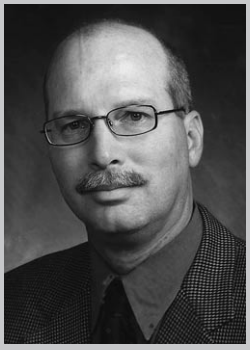

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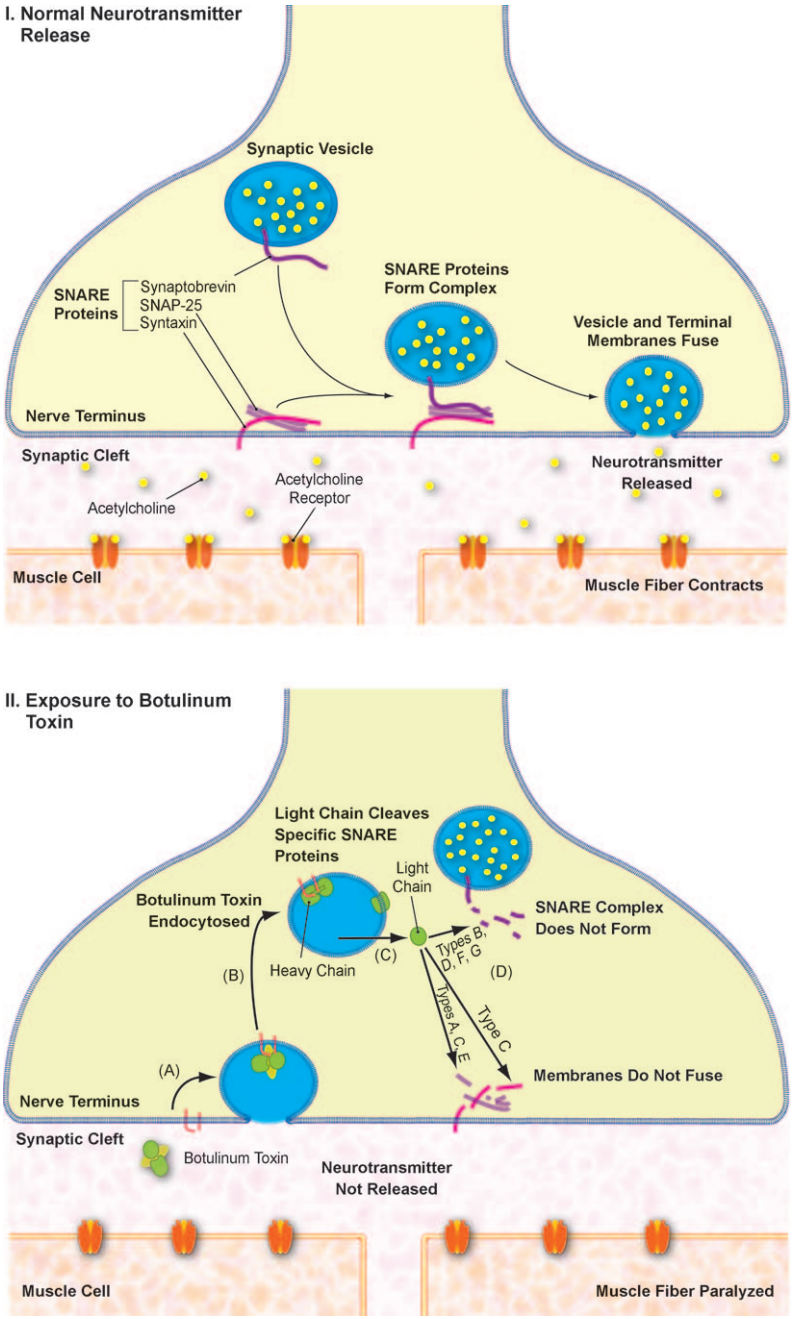


Figure 1. Mechanism of action of BoNTs. I) Normal neurotransmitter release. II) Individual stages of BoNT-intoxication including: A) neurotoxin binding to cell-surface receptors, B) endocytosis into an intracellular vesicle, C) HC-mediated translocation of the LC into the cytosol, and D) proteolytic cleavage of specific SNARE proteins. BoNT/B, /D, /F, and /G cleave synaptobrevin; BoNT/A, /C, and /E cleave SNAP25; BoNT/C also cleaves syntaxin.

that both BoNT/A and BoNT/B bind synaptic protein–vesicle complexes in a similar fashion, and consist of several contacts with such complexes as SV2, synaptotagmin I, synaptophysin, VAMP2, as well as vacuolar proton pumps.[22] Synaptotagmin II, a protein receptor for BoNT/B, has an exposed domain in the lumen of vesicles, thus implicating the possible role of vesicles in the entry mechanism. Exocytosis would place synaptotagmin on the exterior of the nerve cell for a brief time, during which toxin binding to its receptor(s) would occur and both synaptotagmin and BoNT/B would be internalized during membrane retrieval. To support this theory, labeled derivatives of synaptotogmin antibodies have been utilized to monitor membrane retrieval and reformation of intraneuronal vesicles.[23] A recent alternative proposal based on structural studies suggests the putative receptor used by BoNT/A as being the synaptic vesicle protein SV2, thus implying a common use of vesicle recycling for entry of the BoNTs.[24,25]

2.2. Translocation

Since the BoNT substrates reside in the cytosol of the cell, the LC protease must first escape the endosome before cleavage of the SNARE protein can occur. It has been proposed that there is a pH-dependent structural rearrangement of BoNT inside these acidic compartments which allows its entry into the cytosol,[26] a process common among several other bacteria. In general, translocation is believed to take place in such a way that buried endosomal domains are exposed as the pH value decreases.[14] These domains can then facilitate penetration of the lipid bilayer in a fashion that promotes translocation of the light chain into the cytosol. Such a mechanism has been investigated by pretreatment of neuromuscular junctions with chloroquine, a small molecule that can effectively and specifically raise the endosomal pH value.[27] This tactic represents the first nonpeptidic approach for BoNT antagonism by preventing escape of the toxin from the endosome.

The notion that BoNT is internalized by a pH-induced translocaton process is now widely accepted, although the exact nature of the membrane penetration has remained elusive. In an effort to clarify this mechanism, studies have been conducted in which the change in resistance of artificial membranes was measured as a function of the location of the toxin.[28] An additional proposition is that the heavy chain of BoNT can act both as a channel and as a chaperone. It has been shown that BoNT/A and BoNT/E form ion channels in phospholipid bilayers and PC12 cell membranes under conditions similar to those believed to exist in vivo.[29] A recent single-molecule conductance study of BoNT dynamics has shown that discrete intermediate steps exist in translocation, which are characterized by three major events:[30] 1) a closed state, where the BoNT channel is closed; 2) an occluded state, where the partially unfolded LC is trapped within the channel; and 3) an unoccluded state, which is accompanied by the release of the LC. It was shown for BoNT/E that trypsin-activated proteolysis of the LC domain

from its HC chaperone during the occluded stage is mandatory for translocation, a result that is in contrast to that of BoNT/A.[30] By using similar techniques, an additional study has highlighted the role of the disulfide bridge in maintaining the conformational integrity throughout the entire translocation process: premature reduction of the disulfide bridge or substitution of the disulfide with an amide linkage compromised translocation.[31] Hence, cleavage of the LC from the HC only occurs after complete translocation of the LC.

2.3. Catalysis by the Light Chain and Cleavage of SNARE Proteins

SNARE proteins are key components in the fusion of synaptic vesicles with the plasma membrane. Cleavage of SNARE proteins by BoNT inhibits the release of acetylcholine at the neuromuscular junction, thereby resulting in loss of neurotransmission.[13,14] Studies have indicated that cleavage of individual SNARE proteins does not prevent formation of the SNARE complex, but results in a nonfunctional complex where coupling between Ca^{2+} influx and fusion is disrupted.[32] Ca^{2+} ions are fundamental to the process of BoNT-dependent inhibition of neurotransmitter release. It has been shown that increasing concentrations of Ca^{2+} ions in the synaptic terminal partially reverses the effect of BoNT/A.[33]

The light chains of the BoNTs are zinc-dependent metalloproteases that contain the common zinc endopeptidase motif His-Glu-Xaa-Xaa-His within a highly conserved 20 amino acid segment.[34] Each BoNT serotype cleaves one of the three SNARE proteins [synaptobrevin (vesicle-associated membrane protein, VAMP), SNAP25 (synaptosomal associated protein of 25 kD), and syntaxin] which are critical for vesicle fusion and acetylcholine release. VAMP is the target for BoNT/B, /D, /F, and /G, whereas SNAP25 is the target for BoNT/A and BoNT/E. Serotype C can cleave both SNAP25 and syntaxin.

Substantial advancements in understanding the stepwise mechanism of BoNT/A LC (LC/A) recognition and SNAP25 cleavage have been made within the past few years. As detailed in Figure 2, this multistep pathway proceeds through a series of binding interactions between the LC and SNAP25.[35–37] These interactions lead to both the proper alignment of the substrate within the enzyme active site and conformational changes, which ultimately facilitate cleavage of SNAP25. Notably, the stepwise mechanism of LC/E recognition and SNAP25 cleavage has also recently been characterized by using similar techniques.[36] The end result of these studies paints a clearer picture of how the LC domains of BoNTs recognize and cleave SNARE proteins specifically and efficiently, and thus the successful development of potent

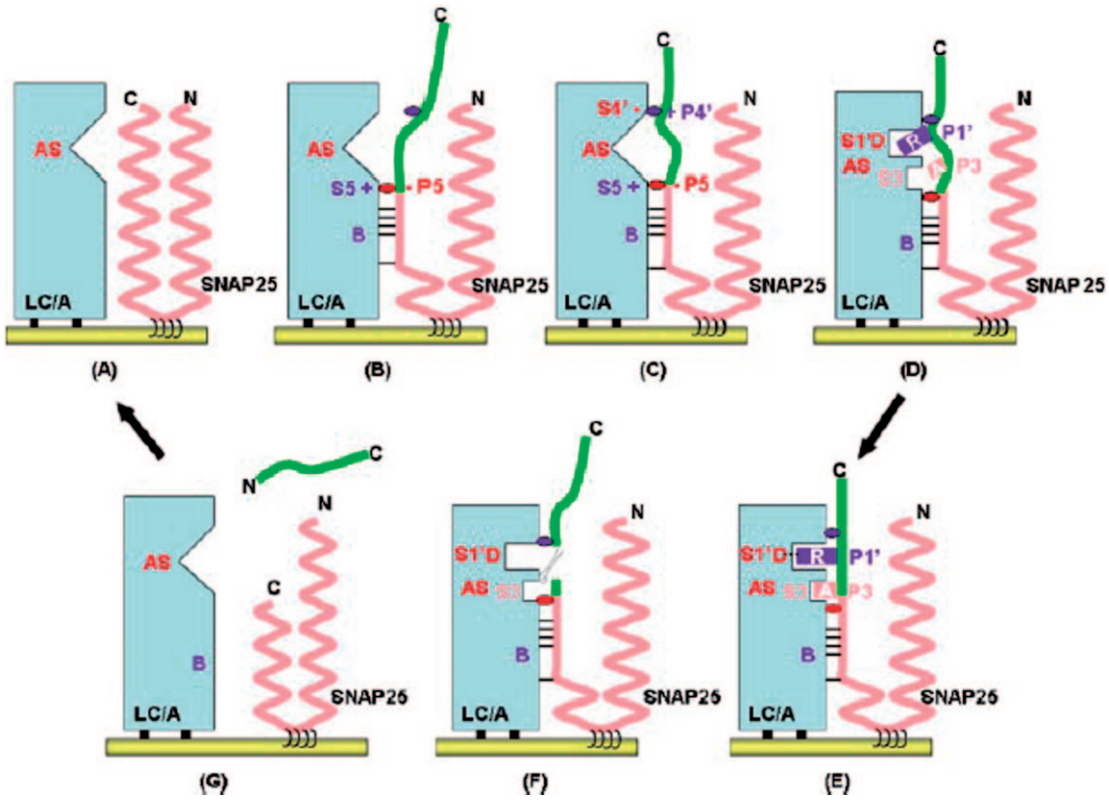


Figure 2. Multistep pathway of the recognition and cleavage of SNAP25 by LC/A. A,B) Plasma–membrane interactions between belt regions of the LC domain and the B region of SNAP25; this binding aligns P_5 (Asp^{193}) to form a salt bridge with P_5 (Arg^{177}). C) Subsequent orientation of the C terminus for $P_{4'}$–$S_{4'}$ interaction (Lys^{201} with Asp^{257}). D) Broadening of the active site to facilitate hydrophobic binding between P_3 and S_3, which aligns $P_{1'}$ (Arg^{198}) in proximity for electrostatic binding with $S_{1'}$ (Asp^{370}). E) The alignment of $P_{1'}$ and P_3 sites within the Zn^{2+} active motif. F) Substrate cleavage. G) After cleavage, release of the C-terminal product allows the active site to return to its original conformation. Reproduced with permission from ASBMB.[37]

LC inhibitors addressing these multiple recognition motifs can be anticipated.

3. Inhibition of BoNT Intoxication

Numerous scientists in academia and industry worldwide are actively pursuing new approaches for the prevention and/or treatment of botulism. There are two types of protective measures currently accepted for treating BoNT poisoning: preexposure prophylaxis with a vaccine, or postexposure countermeasures by administration of sera containing antibodies against BoNTs.

More recently, X-ray structures of the BoNTs have accelerated the rational design of both peptide-based mimics of BoNT substrates and small-molecule inhibitors of the LC domain. Both approaches aim to regulate the effects of BoNT exposure by means of enzyme inhibition. Three steps in the molecular mechanism of cellular intoxication have been exploited as potential therapeutic targets: 1) binding of the toxin to the cell-surface receptors, 2) translocation of the LC into the cytosol, and 3) catalytic cleavage of one of three SNARE proteins. The progress made within each of these individual steps is described below.

3.1. Antagonists of Toxin–Receptor Binding

3.1.1. Active Vaccines

Currently, the US CDC and the US Department of Defense distribute a pentavalent (ABCDE) botulinum toxoid vaccine as a preventative measure against botulism. Even though Park Davis & Co. began development, manufacture, and clinical testing in the 1950s,[38] this vaccine is still classified by the CDC as an Investigational New Drug (IND). The toxoid vaccine contains formalin-inactivated BoNTs (serotypes A, B, C, D, and E) adsorbed onto aluminum phosphate, with thimerosal and formaldehyde added as preservatives. Immunization of an individual requires an initial 0.5 mL of the vaccine to be injected deep subcutaneously followed by three subsequent injections at 2, 12, and 24 weeks, as well as boosters at 12 months and annually thereafter. Upon completion of the initial vaccination series and the first booster at one year, essentially all of the individuals tested in a study had significant amounts of antitoxin, with protective antibody levels persisting for at least 360 days.[39–41]

While this type of pentavalent toxoid has been used to immunize thousands of individuals over half a century, there are a number of potential shortcomings associated with the availability and production of this vaccine. This toxoid-based vaccine is not available to the general public, and is only administered to consenting military personnel that are at risk of exposure and to research laboratory workers that are actively handling the toxin. Furthermore, production of the vaccine is expensive and very difficult because of the requirement for government-regulated facilities, the potential risks associated with cultivating deadly spore-forming bacteria such as *Clostridium botulinum*, and the handling of large amounts of extremely dangerous BoNTs. To overcome these possible problems, a recent study has investigated the potential use of a noncatalytic recombinant form of the toxin as a vaccine candidate.[42] Two single-point mutations within the LC domain rendered BoNT/A inactive and unable to cleave its target protein. Most importantly, the mutated toxin (BoNT/A^{RYM}) maintained the immunogenicity requisite for efficacy as a vaccine. Mice immunized with 1.0 μg of BoNT/A^{RYM} in aluminum hydroxide adjuvant survived subsequent challenges of 10000 times the LD_{50} value of BoNT/A. These new results indicate that recombinant catalytically inactive BoNTs may serve as a viable vaccine in the future.

The majority of recent research efforts have focused on the development of a more widely accessible, cost-effective, and overall safer vaccine based on an individual recombinant domain within the toxin. Studies in the late 1980s with recombinant fragments of tetanus toxin demonstrated that the approximately 50 kDa C-terminal receptor binding domain of the toxin was a good immunogen and able to induce the production of neutralizing antibodies in mice.[43] Since tetanus and botulinum toxins share a high degree of sequence and structural homology,[43–47] a similar strategy for developing the next generation vaccine against botulism was pursued.

Large nontoxic synthetic genes encoding the receptor binding domain of BoNT/A (BoNT/A HC) have been expressed in both *Escherichia coli*[48,49] and *Pichia pastoris*,[50,51] and evaluated for their protection against BoNT-induced death in animals. Initial studies by Middlebrook and co-workers demonstrated that mice receiving two immunizations with crude recombinant BoNT/A HC had partial protection when challenged with BoNT/A at up to 1200 times the LD_{50} value.[48] In a later more detailed study, Smith and co-workers evaluated the efficacy of yeast-produced BoNT/A HC proteins purified to greater than 95% homogeneity.[51] The 50% effective dose (ED_{50}) for these fragments was calculated to be approximately 0.1 μg per mouse. Mice received 1–3 injections with doses ranging from 0.01 to 2.0 μg and then challenged with 100000 times the intraperitoneal LD_{50} value of BoNT/A 21 days after the last injection. Overall, 98% of the vaccinated mice having antibody serum titers of ≥ 1600 survived, while only 14% of the mice having titers of ≤ 100 survived. This study thereby provides a correlation between antibody serum titers and survival.[51] Since disclosure of these promising results, the HC fragments from serotypes A–F have also been investigated.[41,52–57]

The potential power of such recombinant vaccines was highlighted recently in a set of experiments that not only investigated protection in a nonhuman primate model but also utilized an aerosolized toxin exposure model.[58] This model is extremely relevant as it has been postulated that the toxin would most likely to be deployed in this form in a bioterrorist attack. This impressive study demonstrated that vaccination of rhesus monkeys with recombinant BoNT/B HC can provide protection against an aerosol exposure of 50 times the LCT_{50} value of BoNT/B for rhesus monkeys. Furthermore, measurable titers were observed in immunized animals up to two years after vaccination. On the basis of the success of this type of recombinant vaccine in a model

relevant to human exposure, continued research aimed at producing an analogous vaccine for use in humans is ongoing.

Three alternative HC strategies have also been explored: 1) vaccines based on a Venezuelan equine encephalitis (VEE) virus replicon vector system,[59] 2) DNA-based vaccines,[60] and 3) adenovirus-based vaccines.[61] In the first strategy, the BoNT/A HC portion of the neurotoxin was utilized in a VEE replicon particles (VRP) vaccine allowing for the expression of the BoNT/A HC in vivo, thereby eliciting an immune response against the protein.[62] After subcutaneous inoculation with the vaccine, mice were fully protected from a challenge with 100000 times the intraperitoneal LD_{50} value of BoNT/A. In a second strategy, several research groups have investigated vaccines that are based on plasmid DNA that encodes the gene for the BoNT HC protein fused to an Igκ leader sequence[63,64] or BoNT/A HC DNA vehicles predicted to lead to MHC I or MHC II processing.[65] A final strategy investigated the potential of an adenovirus-vectored recombinant vaccine based on the BoNT/C HC.[61] A single dose of the vaccine was capable of eliciting a robust immune response against BoNT/C over a prolonged time period. In general, these three types of vaccines provided protection against BoNTs in animal models to varying degrees, and can therefore be considered as promising candidates for a BoNT vaccine.

3.1.2. Passive Vaccines

The passive administration of antibodies as a defense against biological agents has been investigated as a potential therapy to combat pathogens such as anthrax, BoNT, plague, and smallpox.[66] Currently, the main treatment for human adult botulism is the administration of equine antitoxins (polyclonal antibodies) along with supportive therapy. The CDC distributes two equine antitoxins for the treatment of BoNT/A, /B, and /E. In general, antitoxins are most effective if administered within the first 24 h of the onset of the disease and have been shown to decrease the death rates and shorten the duration of symptoms.[67] Unfortunately, administration of equine serum can cause adverse reactions, such as serum sickness and anaphylaxis, and can potentially lead to hypersensitivity reactions.[68]

Treatment of infant botulism involves passive antibody therapy with human-derived immunoglobulins.[69] Human botulism immune globulin intravenous (BIG-IV) is distributed by the CDC for the treatment of type A and B botulism. When administered promptly, BIG-IV was shown to be safe and effective in reducing the severity of the illness and shortening the length and cost associated with hospital stays.[69] A problem of this method is that the availability of the human-derived immunoglobulins from immunized volunteers is limited.

In general, polyclonal antibodies consist of hundreds to thousands of diverse antibodies that bind multiple epitopes. Immunization of humans or animals with nontoxic forms of a particular antigen allows for the isolation of polyclonal antisera against that immunogen. However, a number of shortcomings may be associated with such polyclonal preparations, including variability from batch to batch,[70] limited supplies,[71] and the potential transmission of infectious diseases,[72] as well as possible side effects linked to the administration of nonhuman sera. In contrast, monoclonal antibodies (mAbs) can be produced in large quantities in high quality and have no infectious risk. Nonhuman mAbs can also be converted or humanized into a less immunogenic format with approximately 90% of its sequence derived from human immunoglobulins.[73]

Monoclonal antibodies against BoNTs may prove to be a valuable therapeutic agent for combating botulinum intoxication.[74] Neutralizing mAbs can be effective if administered as a preexposure prophylaxis to prevent the binding of the toxin to the cell receptors, thereby preventing uptake into the neuronal cell. However, the utilization of neutralizing antibodies as a postexposure prophylaxis may be less effective, mainly because of its short window of application. Analogous to antitoxins, neutralizing antibodies are capable of only sequestering freely circulating BoNTs and consequently become useless once the toxin has undergone endocytosis.[67] Two general techniques have been used to isolate the neutralizing mAbs against several of the BoNTs: 1) immunization of mice with toxoid or BoNT HC followed by standard hybridoma techniques[75–78] and 2) selection of mAbs against BoNTs or toxin fragments by using phage display technology.[79–81]

Studies on the use of mAbs to target BoNTs have primarily focused on serotype A—likely because of its extreme potency and potential use as a bioweapon. To date, there have been no reports of a single mAb against BoNT/A that displays a potent neutralization capability when administered alone. Generally, single mAbs can only neutralize a maximum of 10 to 100 times the LD_{50} value of BoNT/A.[77,82] However, the combination of three mAbs administered as a cocktail has been shown to neutralize 450000 times the LD_{50} of BoNT/A.[83] On the basis of these results, it appears that effective antibody-based therapies for the prevention and/or treatment of botulinum intoxication would require multiple mAbs working synergistically.

3.1.3. Small-Molecule Antagonists of Receptor Binding

Two approaches can be envisioned for the inhibition of toxin–cell interactions: 1) the binding of BoNT by a small molecule, which interfers with the ability of the toxin to interact with cellular receptors and 2) binding of the cellular receptor by a small molecule, thus preventing binding of the toxin. In the former approach, polysialylated gangliosides such as G_{T1b} were shown to be potential receptors for BoNT/A.[84,85] Free G_{T1b} blocks BoNT/A binding to synaptosomes[16] and quenches the tryptophan fluorescence of BoNT/A.[86] More recently, synthetic G_{T1b}-based glycoconjugates that bind the BoNT/A HC were shown to prevent cleavage of SNAP25 in spinal cord cells of rat embryos,[87] thus recapitulating the role of G_{T1b} in HC binding and confirming the potential of targeting the HC as a viable approach for BoNT therapy.

In the second approach, small molecules known to compete with BoNT for the cellular binding site include lectins of both plant and animal origin (*Triticum vulgaris* and

Limax flavus, respectively). In experiments with BoNT/A, these lectins were found to increase the amount of time necessary to cause neuromuscular paralysis in mouse phrenic nerve hemidiaphragm preparations (from 78 min to 128 min), with similar results found for serotypes B–F as well as tetanus toxin.[88] Notably, this was the first report of a universal small-molecule antagaonist of all BoNT serotypes.

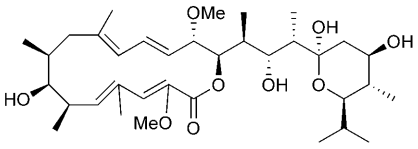
3.2. Antagonists of pH-Dependant BoNT Translocation

The lethality of BoNT is due to suppressed synaptic transmission at the skeletal neuromuscular junctions. Despite variability in the SNARE protein targets by the different BoNT serotypes, all involve holotoxin encapsulation in an endocytotic vesicle and subsequent acidification. This acidification process is needed for BoNT-induced muscle failure. Agents that inhibit acidification have been shown to delay the onset of muscle paralysis in vitro and prolong toxin susceptibility to neutralization with antisera.[27] Thus, the endocytotic vesicle acidification step is an attractive point for inhibiting BoNT activity.

As an example of this approach, both ammonium chloride and methylamine hydrochloride exhibit concentration- and time-dependent antagonism of the onset of neuromuscular blockade by BoNT/A, /B, /C, and tetanus toxin.[89] However, protective effects were observed only when the compounds were added either prior to, or within 10–20 minutes after, challenge by the toxin. Notably, at concentrations that antagonized the onset of BoNT-induced paralysis, the amines showed neither toxin inactivation nor irreversible changes in tissue function. It was therefore presumed that the amines acted solely to antagonize the internalization of the toxins.

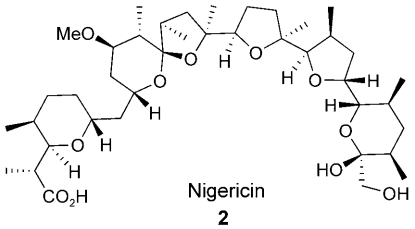
Acidification of endocytotic vesicles requires a vesicular H^+-ATPase that pumps protons from the cytoplasm into the lumen of the vesicle. In 1994, Simpson et al. reported that the ATPase inhibitor bafilomycin A_1 (**1**, Scheme 1) is a universal antagonist of BoNTs A–G as well as tetanus toxin.[90] Bafilomycin A_1 produced a concentration-dependent blockage of neuromuscular transmission without effecting the nerve- or muscle-action potentials. Proton ionophores can also deplete the pH gradient without affecting ATP hydrolysis. For example, nigericin (**2**) and monensin (**3**, Scheme 1), both polyether antibiotics that act as ionophores, increase membrane permeability to the cations H^+, Na^+, and K^+, and act as proton shunts to neutralize pH gradients by blocking endosomal acidification.[91] Although the ionophores showed blockage of BoNT activity at nanomolar concentrations, unfortunately the reverse effect was observed at higher concentrations. An additional H^+-ATPase inhibitor, concanamycin A (**4**, Scheme 1), has recently been studied for inhibition of endosome acidification.[92] Concamycin A prevented cleavage of SNAP25 in pretreated cultured neurons and in those treated up to 15 minutes after exposure to the toxin; however, no protection was observed when addition was made after 40 minutes.

Antimalarial drugs, such as aminoquinolines (Scheme 2), have also been tested for their effectiveness for antagonizing



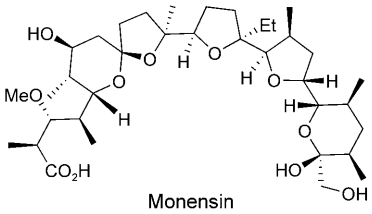
Bafilomycin A_1

1



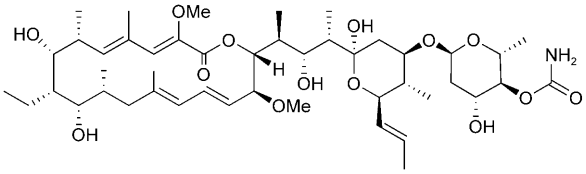
Nigericin

2



Monensin

3



Concanamycin A

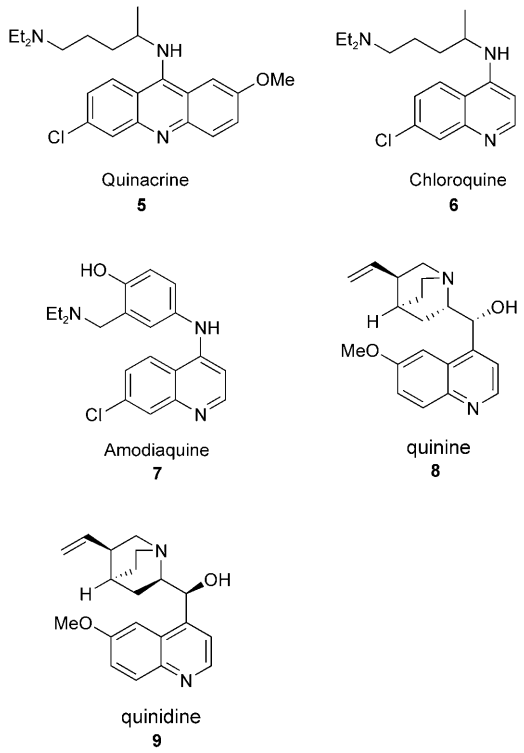
4

Scheme 1. Antagonists of BoNT translocation.

BoNT/A-induced neuromuscular blockage through suppression of endosome acidification.[93] However, the blockage of critical channels responsible for LC release could not be ruled out as a possible antagonistic mechanism. Lastly, Adler et al. have investigated quinacrine and the metal chelator TPEN (*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine) in a "combination therapy" approach, and demonstrated that there was 100% greater protection of BoNT lethality than with either drug alone.[94]

3.3. Inhibition of the BoNT Metalloprotease (LC)

As highlighted above, BoNTs impair neuronal exocytosis arising from specific proteolysis of SNARE proteins, the assembly of which is thought to be intimately linked to membrane fusion and neurotransmitter release.[95] As SNARE hydrolysis is catalyzed by the highly specific BoNT LCs, targeting of the metalloprotease activity provides an additional mode of therapy for BoNT inactivation. The search for BoNT protease inhibitors has primarily focused on the discovery of peptide, peptidomimetics, and small molecules that inhibit BoNT/A and BoNT/B LCs (LC/A and LC/B).



Scheme 2. Aminoquinoline antagonists of BoNT/A-induced neuromuscular blockage.

The common motif in the key proteins VAMP, SNAP25, and syntaxin consists of a nine-residue region that contains a combination of similar chemical features at different points along the adopted α-helical structure.[96] To investigate the role of these conserved segments, a set of peptides were synthesized corresponding to the conserved segments associated with VAMP, SNAP25, and syntaxin (Figure 3).[96] Analysis of this set showed that V2, a synthetic peptide based on VAMP, and S1, a SNAP25-derived peptide, inhibited cleavage of all three SNARE substrates (VAMP, SNAP25, and syntaxin). The two peptides (V2 and S1) showed no effect on neuroexocytosis, but were found to prevent neurotransmitter blockage in cultured *Aplysia californica* neurons. On the basis of these findings it was hypothesized that BoNTs require a dual interaction with their protein targets, namely, they should have a conserved region that is favorable for structural recognition and a cleavage region which is spatially unique to each form.[96]

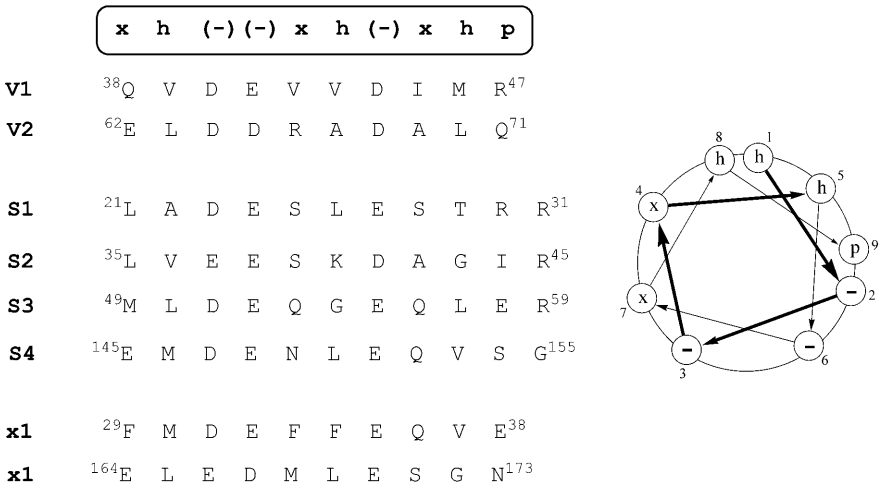


Figure 3. Left: Conserved segments of VAMP, SNAP25, and syntaxin as well as their common chemical features, as shown in the general formula [x h – – x h – x h p], where "x" represents any residue, "–" a carboxylate, "h" a hydrophobic residue, and "p" a polar group. V1 and V2 are synthetic VAMP peptides, S1–S4 are SNAP25 peptides, and X1 and X2 represent the common motif found in syntaxin. Right: Axial projection of the conserved motif. Reprinted with permission from Macmillan Publishers Ltd.[96]

3.3.1. *Peptide Mimics of the BoNT/A Light Chain*

A small collection of peptides based upon the general formula Ac-X_1-X_2-linker-X_3-X_4-NH_2, where the linker was 4-aminobutyric acid and the X_n monomers were a variation of Asp, Glu, Gln, and Arg residues, were designed on the basis of the common structural features of the SNARE complexes.[97] The linker was chosen to alter the conformation of the amino acid backbone in efforts to enhance BoNT recognition. Further efforts extended the repertoire of amino acids to 12 (giving 12^4 different peptides for study)[98] and used positional scanning synthetic combinatorial library (PS-SCL) technology[99] with the aim of improving the deconvolution strategy to identify the optimal peptide.

The compilation of these studies revealed that the most potent LC/A inhibitors were of the general formula Ac-X_1M_2-linker-X_3C_4-NH_2 and C_1-linker-X_2S_3, both exhibiting 75% inhibition. LC/B inhibition of over 80% was observed with peptide libraries of the formula Ac-X_1C_2-linker-X_3C_4-NH_2 and C_1-linker-X_2F_3.[99]

Several years ago, Schmidt et al. recognized the potential for developing BoNT/A inhibitors by exploiting the metalloprotease (LC) properties of the BoNT.[100] Since sulfhydryl groups readily complex zinc, it was reasoned that substitution of key amino acid residues with Cys would provide molecules that inhibit the enzymatic activity of BoNTs. Additional support for the design of such inhibitors was provided by the observations that thiol-containing ligands (for example, dithiothreitol) and even cysteine monomers alone can inhibit LC/A, albeit at high concentrations (>10 mM).

The choice of the modification site varied in the SNAP25 194–200 region (designated as P_4, P_3, P_2, P_1, $P_{1'}$, $P_{2'}$, $P_{3'}$) of the 17-residue substrate N^{α}-acetyl-SNKTRID**EANQRAT**KML-carboxamide, where the residues in bold were separately replaced with Cys (Table 1). Interestingly, those substrates containing Cys substitutions at the P_4, P_3, $P_{2'}$, and $P_{3'}$ sites were hydrolyzed by LC/A. A key finding with these substrates was that no relationship between binding affinity and rate of hydrolysis was apparent.

Table 1: Substrate analogues prepared by substituting cysteine in the P_4 to $P_{3'}$ region of a 17-residue SNAP25 mimic.

Peptide	Partial sequence						
	P_4	P_3	P_2	P_1	$P_{1'}$	$P_{2'}$	$P_{3'}$
	8	9	10	11	12	13	14
SNAP	E	A	N	Q	R	A	T
E8C	C	A	N	Q	R	A	T
A9C	E	C	N	Q	R	A	T
N10C	E	A	C	Q	R	A	T
N10D-C	E	A	D-C	Q	R	A	T
Q11C	E	A	N	C	R	A	T
Q11D-C	E	A	N	D-C	R	A	T
R12C	E	A	N	Q	C	A	T
A13C	E	A	N	Q	R	C	T
T14C	E	A	N	Q	R	A	C

Table 2: Shortened peptide inhibitors of variable cysteine content in the P_2 to $P_{1'}$ region.

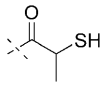
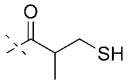
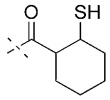
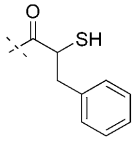
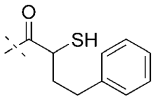
Peptide	Sequence								K_i [μM]
	P_2	P_1	$P_{1'}$	$P_{2'}$					
1	C	Q	R	A	T	K	M	L	190
2	D-C	Q	R	A	T	K	M	L	140
3		C	R	A	T	K	M	L	1.9
4		D-C	R	A	T	K	M	L	1.8
5	N	C	R	A	T	K	M	L	500
6	A	C	R	A	T	K	M	L	150
7	N	D-C	R	A	T	K	M	L	150
8	A	D-C	R	A	T	K	M	L	26
9	C	D-C	R	A	T	K	M	L	110
10		C	D-R	A	T	K	M	L	410

For example, peptides substituting Cys at end positions (P_4 or $P_{3'}$) were observed to have lower K_m values than the native SNAP25 sequence, but had similar k_{cat} values. The inverse relationship was observed when Ala residues closer to the cleavage site (P_3 or $P_{2'}$) were replaced. Therefore, the authors concluded that substrate discrimination at the complementary binding sites must occur at the catalytic step.[100]

Additionally, a number of LC/A inhibitors were identified with Cys substitutions at the P_2, P_1, and $P_{1'}$ sites (Table 1). In all cases—except for Arg—significantly tighter binding than the native SNAP25 sequence was observed when $P_{1'}$ was replaced. In general, Cys substitution at the P_2 and P_1 sites resulted in good inhibition, whereas replacing the $P_{1'}$ Arg residue resulted in an inactive peptide capable of neither hydrolysis nor inhibition. This finding highlighted the critical requirement for the $P_{1'}$ Arg residue for strong binding. Interestingly, replacement of the P_1 site with D-Cys (Q11D-C) gave the strongest inhibition.[100] Peptides that exhibited significant inhibition were then truncated to give octapeptides beginning with the P_2 site. Substitutions at both the P_2 and P_1 sites were also made to assess the importance of the Cys placement within this two-residue region. As shown in Table 2, it is clear that shorter peptides can bind BoNT/A with significantly higher affinity than can the native SNAP25 substrate. It was suggested from these findings that two critical requirements must be met in designing peptides LC/A inhibitors: a P_1 thiol group for binding the zinc atom at the active site and a $P_{1'}$ Arg residue for tight binding of the substrate.[100]

Second-generation peptidomimetic LC/A inhibitors focused on Cys analogues at the P_1-position.[101] Two design strategies were employed: 1) the thiol group was retained on the β carbon atom, while the α-amino group was substituted by various other functional groups; 2) the β carbon atom was modified with alternative functional groups, and the thiol group was moved to the α carbon atom (Table 3). After synthesis of the monomers, each mimetic was individually conjugated at the amino terminus of the hexameric peptide RATKML. The most effective peptidomimetic incorporated the L stereoisomer of the 2-mercapto-3-phenylpropionyl group (mpp) at the N-terminus (compound **13b**), and represented the first LC/A inhibitor with a K_i value in the nanomolar range (competitive inhibition, $K_i = 330 \pm 50$ nM).

Table 3: N-Terminal structures of cysteine mimics of the sequence C_{mim}RATKML$_{(amide)}$.[a]

	Stereoisomer	K_i [μM]
	**10a**	1
	10b	30
	**11a**	50
	11b	20
	**12a**	70
	12b	100
	**13a**	8
	13b	0.3
	**14a** + **14b**	2

[a] The stereoisomer eluting first by HPLC is designated **a**, and the second eluting stereoisomer designated **b**.

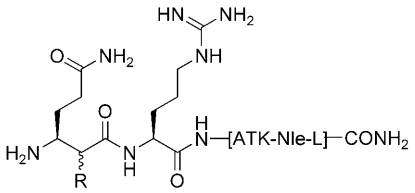
Additional analysis of **13b** was performed by varying the amino acid composition in conjunction with extension or truncation of the central hexamer region. It was shown that the peptide mpp-RATKM contains the minimal structural requirements for high binding affinity (Table 4). Closer analysis of Table 4 underscores the importance of the P_1 Arg residue, thus supporting earlier studies that attribute the specificity of the inhibitor/substrate–enzyme interaction to this key residue.[100] However, it must be noted that current understanding of the BoNT/A catalytic mechanism recognizes a much more complex structural requirement in achieving potent inhibition.[35–37]

In an effort to characterize the conformation of the enzyme–substrate complex of BoNT metalloprotease, Rich and co-workers have developed isosteric di- and tripeptide derivatives consisting of transition-state analogues of the

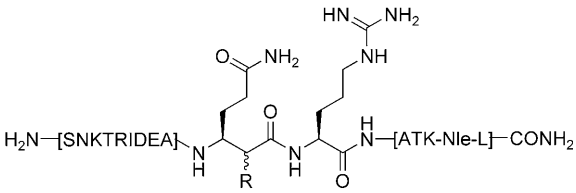
Table 4: The effect of extended and truncated versions of $C_{mim}RATKML_{(amide)}$ on the K_i value.

Inhibitor	K_i [μM]	Inhibitor	K_i [μM]
mpp-R	60	mpp-RATKMLGSG	0.3
mpp-RA	60	mpp-RATKAL	0.7
mpp-RAT	30	mpp-RATAML	3
mpp-RATK	4	mpp-RAAKML	0.7
mpp-RATKM	0.3	mpp-RVTKML	2
mpp-RATKML	0.3	mpp-KATKML	> 300

cleavable sites for both LC/A and LC/B.[102,103] The hydroxyethylene or thioethylene analogues were incorporated into longer peptides corresponding to the cleavage products associated with both serotypes.[104,105] In the case of BoNT/A, the modified peptides were incorporated into a 17-mer peptide analogous to the 187–203 region of SNAP25 (**16a** and **16b**, Scheme 3)[104] as well as a shorter, 7-mer peptide (**15a** and **15b**) based on previous studies.[101] The peptides displayed significant inhibition (K_i = 200–400 nM), with little correlation between peptidomimetic length and inhibitory activity, clearly indicating that much shorter peptides can be utilized for LC/A inhibition. Studies by Schmidt and Stafford further corroborated this finding (**13b**, BoNT/A K_i = 330 nM).[101]



15a R = OH
15b R = SH



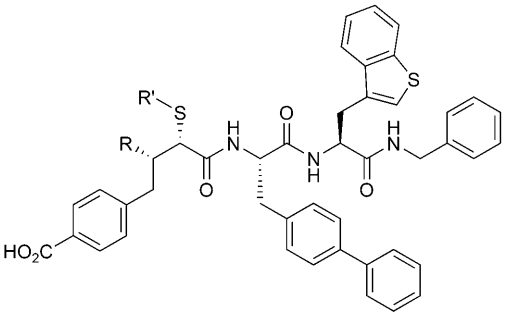
16a R = OH
16b R = SH

Scheme 3. Peptide mimics of SNAP25 that utilize an α-hydroxy or α-thioethylene group at the cleavage site. Mimics containing the α-thioethylene moiety exhibit the strongest BoNT/A metalloprotease inhibitory activity (K_i in sub-μM range).

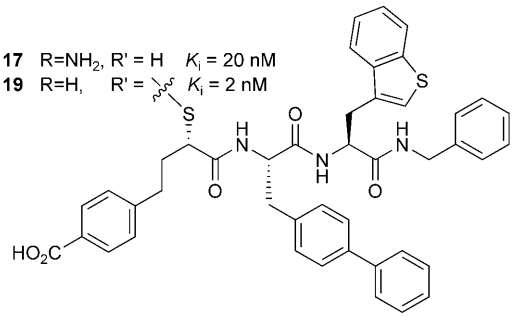
3.3.2. *Peptide Mimics of the BoNT/B Light Chain*

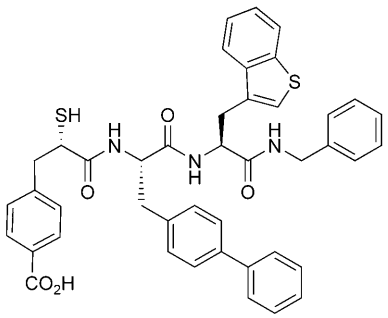
Using a combination of molecular modeling and structural data, Rocques and co-workers conducted the first study of the structure–activity relationship of LC/B inhibitors based on β-aminothiol derivatives.[106] The choice of β-aminothiol was due to its characterized peptidase inhibitory activity of tetanus toxin, a structural relative of BoNT/B with an identical VAMP cleavage site.[107,108] In the study, two libraries of pseudotripeptides were prepared, each aimed at optimizing the recognition of the $S_{1'}$ and $S_{2'}$ subsites.[106] After testing several side chains in the $P_{1'}$-position, it was found that a phenyl group bearing a carboxylate at the 4-position gave optimal recognition.

In regard to optimizing the $P_{1'}$ residue, bulky aromatic ligands were recognized as favorable since the deep and hydrophobic $S_{1'}$ subsite presents itself in the crystal structures of LC/B and holo-BoNT/B[109,110] and because of the presence of phenylalanine in the native LC/B substrate. The optimization of the $S_{2'}$ recognition indicated a preference for a bicyclic heteroaromatic residue. Interestingly, this residue in natural VAMP is Glu; therefore, the natural recognition at this subsite is not ideal and may be the reason why catalytic activity is independent of modifications at this position. The pseudopeptide to emerge from these studies (**17**, Scheme 4) exhibited significantly enhanced potency over any peptide at that time (K_i = 20 nM).[106] Further optimization of the P_1 residue of the pseudotripeptide inhibitors have resulted in ligands with slightly more potency than **17**, for example,



17 R=NH_2, R' = H K_i = 20 nM
19 R=H, R' = K_i = 2 nM





18 K_i = 3 nM

Scheme 4. Thiol- and disulfide-tripeptide surrogates with potent LC/B cleavage activity.

compound **18**, which has one methylene group less (Scheme 4).[111] Amazingly, compound **19**, a closely related disulfide derivative of **17**, exhibited an up to tenfold enhanced inhibition of LC/B.[111]

Attempts to increase the inhibition of LC/B by embedding either an α-hydroxyamido or an α-thioamido moiety within 35-mer VAMP analogues at the Gln-Phe connection resulted in moderate inhibition (low micromolar K_i values), being nearly 25% less potent than tripeptide derivatives. However, a clear drawback in this design stems from the additional substituted methylene group at the site of modification, which makes the backbone less recognizable by LC/B.[106]

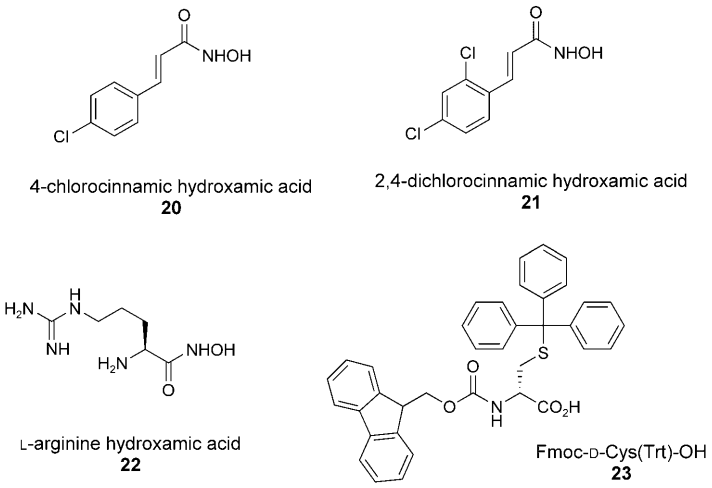
3.3.3. *Biochemical Techniques*

Phage display has been utilized for the generation of peptides capable of inhibiting multiple BoNT serotypes.[112,113] Three different peptides were identified and found to inhibit protease activity in vitro.[113] However, of all the LC serotypes studied, only moderate binding by the peptides to LC/A, /B, and /E was exhibited (250 μM for complete inhibition). Nonetheless, this study provides an example of the utility of phage display for identifying lead peptides that can potentially be exploited both for further clarification of the molecular requirements necessary for BoNT inhibition and for insight into the BoNT mechanism of action. In a more recent study, phage display of VAMP variants was used to determine substrate recognition domains important for LC/B binding and catalysis.[112]

Messenger RNA (mRNA) display also has been used to identify LC/A inhibitors.[114] A constant region, CRATKML, corresponding to the C terminus of SNAP25 was maintained, and libraries of four random amino acids attached to this region gave a possible 160000 sequences; the overall formula was MXXXXCRATKML, where X was a random amino acid. Several peptides were identified after three rounds of screening. Perhaps surprising was the affluence of nonpolar residues within the sequences. The peptide MWMTSCRATKML was among the most active inibitors, with K_i values in the low micromolar range, a potency significantly greater than those identified by phage display (K_i values in the sub-mM range). The mRNA display technique therefore represents a potentially effective approach to identify new peptides for BoNT recognition as well as for identifying common chemical themes within the screened peptides.[114]

3.3.4. *Zn^{2+} Chelating Ligands*

A recent approach aimed at identifying novel small-molecule inhibitors of LC/A has relied on an assay based on high-throughput fluorescence resonance energy transfer (FRET). During validation of this assay, the single modified amino acid, L-Arg hydroxamic acid (**22**, Scheme 5), was shown to modestly inhibit LC/A ($K_i = 60$ μM).[115] Given the native cleavage site between residues Gln197 and Arg198 of SNAP25, inhibition by the Arg derivative was somewhat expected; however, the similarity in inhibition between the D and L isomers was not. It was suggested that the stereochemical configuration was not important for this class of inhibitors and that the hydroxamate moiety dominated the inhibitor activity, most likely through chelation of Zn^{2+} ions within the enzyme active site. Indeed, a recent crystal structure of LC/A complexed with L-arginine hydroxamate has indicated an intermediate conformation of the active site, with an intact coordination of the Zn^{2+} ion to the hydroxamate region and with Asp369 clearly bound to the Arg side chain.[116]



Scheme 5. Structures of LC/A metalloprotease inhibitors.

On the basis of these early studies, the coupling of recognition scaffolds to hydroxamate moieties was envisioned to provide potent LC/A inhibitors. Therefore, a 150-member library of hydroxamic acids was prepared by a facile two-step procedure starting from the corresponding carboxylic acids.[117] The initial screen showed 4-chlorocinnamic hydroxamate (**20**, Scheme 5) to be a promising lead, with an IC_{50} value of 15 μM, thus prompting a structure–activity study that resulted in a 2,4-dicholoro-substituted derivative (**21**, Scheme 5). Compound **21** is the most potent nonpeptidic inhibitor of LC/A reported to date ($K_i = 0.30 \pm 0.01$ μM) and acts as a competitive inhibitor of LC/A, thus disproving a recent hypothesis that a minimum of ten carbon atoms is required for effective inhibition of BoNT.[118,119] Crystal structures of the LC/A complexed with the inhibitors L-Arg hydroxamate, 4-chlorocinnamic acid, and 2,4-dichlorocinnamic acid have indicated a somewhat flexible enzyme active site, thereby underscoring the likelihood that substrate specificity is driven by exosite interactions.[120]

The metal chelator TPEN has been extensively studied for its antagonistic properties toward BoNT catalysis in mouse phrenic nerve hemidiaphragm preparations and in a mouse model.[85,121,122] However, the lack of specificity this compound displays for BoNT over other metalloproteins limits its clinical utility. A more recent study has shown that EDTA irreversibly inhibits BoNT/A and BoNT/E protease activity in cell-free assays but, unlike TPEN, shows no activity in cultured neurons.[123] While these findings support previous studies that suggest EDTA-induced LC denaturation occurs in a Zn^{2+}-independent fashion,[124,125] it highlights the variability between in vitro and in vivo experiments. An additional discrepancy in the results between in vitro screening assays

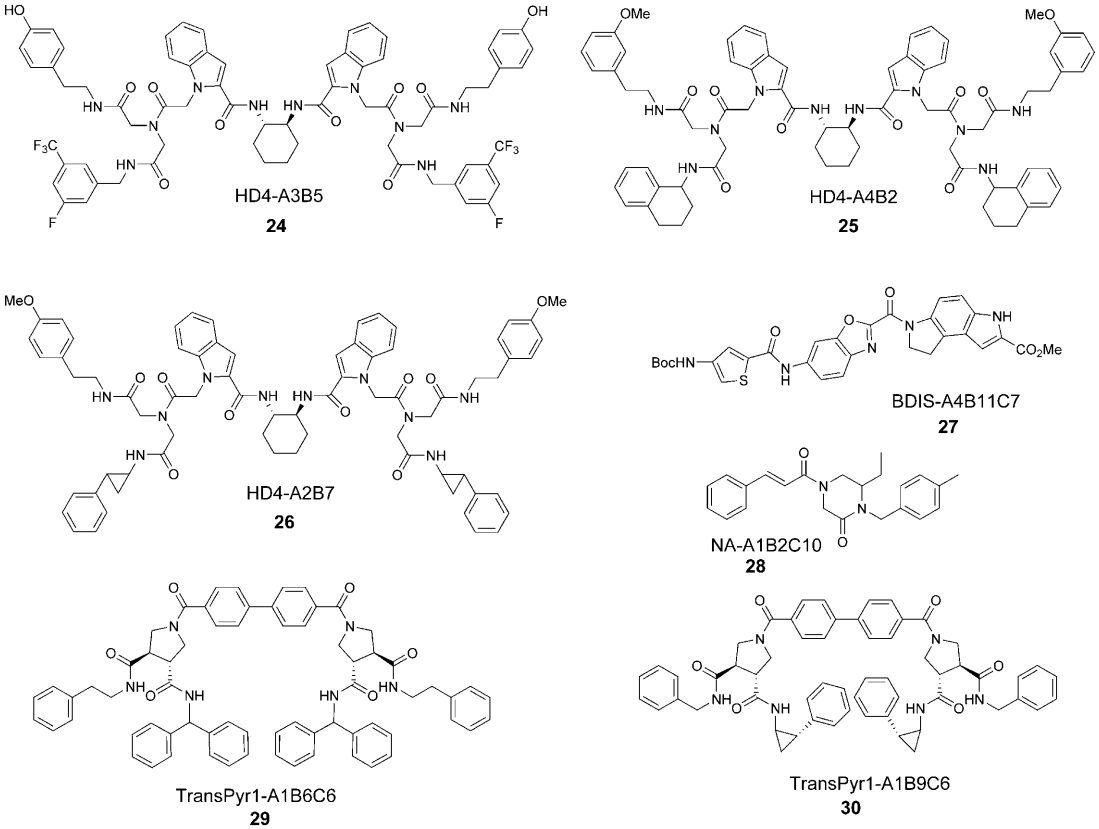
and mouse bioassays was very recently accentuated in a series of cellular, animal, and in vitro FRET assays where lead compounds from a pool of about 66000 compounds aimed at disrupting protein–protein interactions were analyzed.[126] This FRET assay and subsequent cell study identified seven compounds (Scheme 6) that exhibited promising inhibition and these were advanced through in vivo testing. Of the seven hits, all lacked a characteristic zinc-chelation moiety, thus indicating additional LC/A recognition motifs may play a role in their activity. Interestingly, the most efficacious inhibitors, NA-A1B2C10 (**28**, Scheme 6) and 2,4-dichlorocinnamic hydroxamic acid (**21**, Scheme 5), share structural simplicity and possess similar functional regions (cinnamic acid and amide moieties). Unfortunately, in vivo analysis of these two compounds indicated that current cellular models are unable to predict in vivo efficacy and that research aimed at developing better cellular models is critical. However, the similarity between **21** and **28** is promising, and optimization of **28** may lead to more potent LC/A inhibitors.

In a surprising finding, the simple protected amino acid Fmoc-D-Cys(Trt)-OH (**23**, Scheme 5) commonly used in peptide synthesis was found to competitively inhibit LC/A ($K_i = 18$ μM).[127] Docking of **23** into the enzyme active site indicated significant binding between the fluorenyl protecting group and a hydrophobic pocket as well as favorable electrostatic interactions between the carboxylate group and several proximal positively charged residues. Interestingly, no interaction was predicted between any portion of the small molecule and the critical zinc ion in the active site. In addition, the potency of **23** was investigated in a cellular model, and at a concentration of 60 μM provided complete protection of SNAP25 cleavage in Neuro-2a cells.

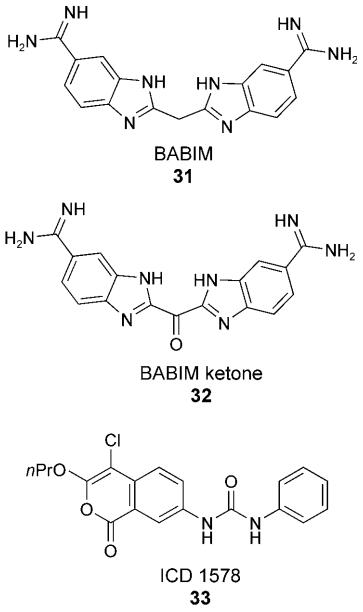
3.3.5. *Small-Molecule Inhibitors of the BoNT/B Light Chain*

The pseudotripeptide study by Roques and co-workers has remained the most detailed analysis of a small molecule capable of disrupting the binding of VAMP to LC/B, thereby inhibiting catalysis.[106] Over the past decade, several accounts addressing a small-molecule approach for inhibiting the action of LC/B have surfaced. A collaboration between the Rich and Stevens research groups has demonstrated that bis(5-amidino-2-benzimidazolyl)methane (BABIM, **31**; Scheme 7) inhibits BoNT/B-induced cleavage in the low micromolar range. The keto version of BABIM **32** (Scheme 7) was found to be slightly more potent.[128] The structural recognition of BABIM was confirmed by comparison studies with the exceptional zinc chelator 1,10-phenanthroline, with which LC/B inhibition was nonexistent.

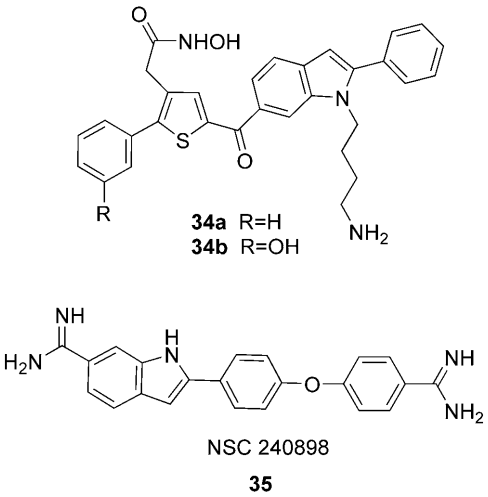
Earlier research focused on the development of potential LC/B inhibitors without the aid of structural data.[129] The foundation for the design was in the structural similarity it shared with a previously studied but weakly active phosphoramidon derivative. The compound of study was the coumarin derivative ICD1578 (**33**, Scheme 7). Moderate inhibition, with an IC_{50} value of 28 μM, was observed in this study. Notably, the zinc metalloprotease inhibitor captopril exhibited less than 10% inhibition at millimolar concentrations,



Scheme 6. Structures of LC/A metalloprotease inhibitors discovered from a high-throughput screening assay.



Scheme 7. Chemical structures of LC/B metalloprotease inhibitors.



Scheme 8. Structures of small-molecule LC/A inhibitors identified by structure-guided screening.

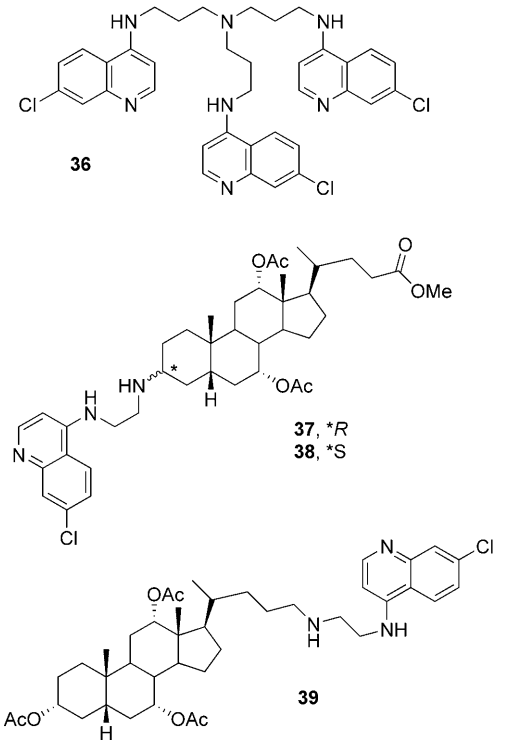
thus highlighting the presence of key recognition moieties in the molecule.[129]

3.3.6. *Structure-Guided Screening and Pharmacophore Development*

An additional approach to the identification of small-molecule inhibitors of LC/A includes the screening of the National Cancer Institute (NCI) Diversity set. From this, a series of 4-aminoquinolines were found to slow BoNT/A-induced effects on neuromuscular transmission.[119] A number of compounds possessing greater than 50% inhibition (at 20 μM concentration) were identified and modeled with the BoNT/A crystal structure[45] in computational studies for predicting common pharmacophore scaffolds. A further in silico screen of 2.5 million compounds was later conducted with the same goal.[118] Optimization techniques centered around molecular dynamics simulations of a complex formed between the zinc endopeptidase and the inhibitor by using a molecular dynamics simulation (the cationic dummy approach, CaDA).[130] After extensive structure-guided modification, a competitive inhibitor (**34a**, Scheme 8) with a K_i value of 12 μM was found.[118] Further analysis and optimization of **34a** has resulted in a slightly more potent derivative incorporating a 3-hydroxyphenyl moiety (**34b**, Scheme 8).[131] The success of these studies validates the utility of such molecular dynamics simulations in the development of BoNT inhibitors.

Recently, the potent LC/A inhibitor mpp-RATKML[101] was used in a similar molecular docking study to identify the key molecular components required for inhibition.[132] In this extensive study, initial attempts to generate satisfactory binding conformations when mpp-RATKML was docked into the enzyme structure obtained from a crystal structure of LC/A[133] led to concern as to whether the crystal structure of LC/A was consistent with its bioactive form in solution. Furthermore, comparison of the empirical data of mpp-RATKML with those of mpp-KATKML indicated a greater than 1000-fold loss in potency for the Lys-substituted peptide, whereas modeling with the crystal structure data indicated that the low-energy conformations of both peptides maintain similar interactions in regard to the cationic nature of the protonated Lys or Arg residues. Another conflicting result was the placement of the thiol moiety in mpp-RATKML within the binding cleft of the natural ^{198}RATKM^{203}L sequence of SNAP25: the sulfur atom was approximately 7 Å from the zinc ion, thus eliminating the possibility of S–Zn^{2+} coordination. This finding prompted a molecular dynamics study in which favorable surface conformations between mpp-RATKML and LC/A were used. In accordance with empirical evidence, a model was developed whose conformation of the binding site showed a strong energetic preference for Arg over Lys. Additional requirements in the model were the placement of the thiol group in mpp-RATKML in proximity to the Zn^{2+} ion (for coordination), and elimination of the hydrophobic–polar clash of the isobutyl group of Leu and the phenyl groups of mpp with water. Several key small requirements for a potent small molecule were postulated from the molecular dynamics study. These include an aromatic group connected by a planar cationic group (for example, guanidinium), a protonated amino group, at least two planar regions separated by a distance of 6.5–13 Å, and an overall molecular length of approximately 23 Å. A rigorous search for nonpeptidic small molecules in the National Cancer Institute's Open Repository identified several small molecules as potential LC/A inhibitors. The most outstanding was NSC240898 (**35**, Scheme 8), which exhibited significant dose-dependent protection of SNAP25 in chicken neurons as well as a good cellular tolerance up to 40 μM.[132]

Four inhibitors were identified (Scheme 9) from the refined, eight-component pharmacophore model for in vitro testing with LC/A, and were synthesized.[134] All four compounds exhibited IC_{50} values in the low micromolar range (3–17 μM). The unique aspect of three of the novel inhibitors was



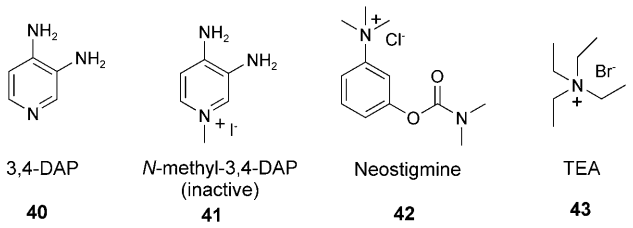
Scheme 9. Aminochloroquinoline (ACQ) based inhibitors of the LC domain of BoNT.

the incorporation of an additional recognition motif in a steroidal region to act as a hydrophobic anchor at the edge of the binding cleft. However, the most potent of these was that lacking the steroid but containing a tris(aminochloroquinoline) motif (**36**, Scheme 9).

3.4. K^+ Channel Blockers

K^+ channels are vital to virtually all types of cells[135] and represent a remarkably diverse group of ion-channel structures. The first elucidated K^+ channel was the classical voltage-activated channel found in the squid. Other channel types include hyperpolarizing voltage channels that are controlled by intracellular metabolites and secondary messengers, transient outward currents (A currents), and large conductance Ca^{2+}-sensitive, K^+ channels (BKCa channels) in which neurons are characterized by calcium-activated K^+ currents that aid in repolarization and firing. The BKCa channels are composed of two subunits, the pore-forming α subunit and the regulatory β subunit, and are typically believed to be sensitive to tetraethylammonium (TEA, **43**, Scheme 10). The prospect of using BKCa channel blockers in BoNT therapy lies in the ability of the channel blocker to induce calcium influx, which prompts acetylcholine flux from the cell. Such agents would thus be particularly applicable to reversing BoNT-induced paralysis. Indeed, TEA has been shown to reverse BoNT-induced paralysis in vitro[136] and in animal models.[137,138]

Considered the best K^+ channel blocker for botulism poisoning, 3,4-diaminopyridine (3,4-DAP, **40**; Scheme 10) is



Scheme 10. Chemical structures of K^+ channel blockers and the inactive *N*-methyl-3,4-DAP$^+$ derivative.

highly effective in antagonizing muscle paralysis following BoNT/A exposure in vitro, and is the least toxic of the currently available K^+ blockers.[139] BoNT-paralyzed rat diaphragm muscle was shown to be rapidly reversed by 3,4-DAP in twitch tension experiments, with prolonged movement at least eight hours after administration of the drug. Adler et al. combined in vitro and in vivo recording techniques to examine 3,4-DAP action in the rat extensor *digitorum longus* muscle after local BoNT-induced paralysis, and revealed a significantly increased twitch tension in the intoxicated muscle with no loss of mobility with time or in repeated applications.[140] One key concern of the study was the constant administration of 3,4-DAP during the intoxication period because of the rapid clearance of this compound. To address this requirement, osmotic minipumps have been utilized for 3,4-DAP delivery.[141]

Although 3,4-DAP is an impressive compound for BoNT treatment, there are notable drawbacks in its use. Unfortunately, drug efficacy is limited primarily to BoNT/A. Additionally, while an increase in muscle strength is the most substantial indication of drug action, 3,4-DAP results in miniscule improvement for the respiratory muscles and no return of spontaneous ventilation. Lastly, a critical side effect of 3,4-DAP are seizures, which most likely result as a consequence of the drug's ability to penetrate the blood–brain barrier. To combat some of these difficulties, combinations of 3,4-DAP with neostigmine (**42**, Scheme 10) and TEA have been tested.[139] The combination approach was aimed at lowering the dosage of 3,4-DAP, and thus its toxic effects, while achieving increased acetylcholine efflux over that occurring in individual drug administrations. Combination therapy of neostigmine with 3,4-DAP was anticipated to boost acetylcholine levels through neostigmine inhibition of acetylcholinesterase in peripheral tissues. Unfortunately, no additional benefit was observed. In the case of TEA combinations, diminished effects of 3,4-DAP were observed, thus suggesting that the action of TEA on the nicotinic ion channel counteracted any beneficial action of TEA on transmitter release. Although both cases were unsuccessful, the concept of "combinatorial" channel blockers remains an area of optimism in the treatment of BoNT poisoning.

Adler et al. have provided additional insight into the effects of 3,4-DAP by determining the location of binding at the nerve terminal.[139] A cationic 3,4-DAP derivative, 3,4-diamino-1-methylpyridium (**41**, Scheme 10), was also tested in rat phrenic nerve hemidiaphragms and showed little or no activity up to millimolar concentrations. The inability of the pyridinium derivative to augment muscle tensions by binding

to the K^+ ion channel suggests that 3,4-DAP action must take place on the internal membrane surface where a charged species is not favorable.

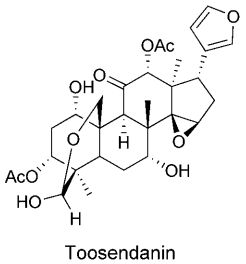
The efficacy of K^+ channel blockers in antagonizing or reversing the action of BoNT/A is generally attributed to their ability to induce the Ca^{2+} efflux by inhibiting voltage-dependent K^+ currents. Further efforts to develop optimal derivatives of 3,4-DAP with increased efficacy and reduced toxicity are of significant interest. However, a lingering concern with the viability of this approach for the treatment of BoNT intoxication is that a K^+ channel blocker must be administered over a period of weeks to months to show any protection.

3.5. Therapeutics with an Undefined Mechanism of Action

Theaflavins, the active ingredient produced from the fermentation of green tea into black or oolong tea, can polymerize to give, among other complex polyphenols, a thearubigin fraction that is active against the effects of BoNT/A, /B, and /E.[142] Nishimura and co-workers have utilized rat cerebrocortical synaptosomes exposed to ^{125}I-labeled BoNT in attempts to elucidate the mechanism of action of the thearubigin fraction.[143] Unfortunately, no information as to the identity of the active molecule(s) could be obtained and the sole conclusion was that the thearubigin fraction functions by simple binding of the toxin.

Limonoids are tetranortriterpenoids derived from euphane or tirucallane triterpenoids that are characterized by an intensely bitter taste.[144] Structurally, the molecules are composed of a 4,4,8-trimethylfuranylsteroid skeleton. Limonoids from the tree *Melia toosendan* have been known for many years to possess potent antihelmintic properties.[145] A major constituent in the limonoid family of *M. toosendan* is toosendanin (**44**, Scheme 11), which is known to have multiple modes of action in insects.[146]

While liminoid natural products remain an area of interest in pest management research, a number of publications have emerged over the past two decades detailing toosendanin's effect on neurotransmitter release in motor nerve terminals.[147] Collectively these studies characterize toosendanin as a selective presynaptic blocker that inhibits quantal release of acetylcholine.[148] The blocking effect of toosendanin is preceded by a Ca^{2+}-dependent facilitator phase, a phenomenon in direct contrast with that observed for BoNT. A noteworthy observation in one of BoNT studies with toosendanin was that the tolerance of the neuromuscular junction towards BoNT was enhanced significantly during the facilitatory period.[149]



Toosendanin

44

Scheme 11. Chemical structure of the limonoid toosendanin.

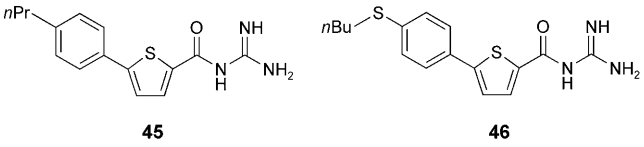
Other studies investigating the mechanism of toosendanin described evidence suggesting that toosendanin causes a decrease, and ultimately disappearance, of Ca^{2+} sensitivity. This effect was responsible for the observed restriction of voltage-dependent K^+ current in neuroblastoma glioma cells.[150] Toosendanin was also found to induce submicroscopic changes in the neuromuscular junction, namely, a reduction in the amount of synaptic vesicles and an increase in the girth of the synaptic cleft.[151] In addition, toosendanin was shown to inhibit the delayed rectifier K^+ channel by intracellular and/or extracellular addition.[152]

The membrane permeability and modulation of ion channels by toosendanin is perhaps due to its ability to span membrane bilayers and alter channel integrity. This action, in the context of BoNT, is important because of the capability of the toxin for forming ion channels in artificial bilayers and in PC12 cell membranes.[153] Additionally, treatment of synaptosomes with toosendanin results in resistance to BoNT/A-mediated cleavage of SNAP25. When considering the correlation between BoNT translocation and channel formation, these studies suggest that the effect of toosendanin on BoNT may be due to an interference with LC translocation. However, a detailed mechanistic picture of toosendanin action in BoNT antagonism remains unresolved.

4. Agonism of BoNT/A

4.1. Small-Molecule Activators

While BoNT has a history of treating medical ailments, it has also been found to elicit an immune response, where neutralizing antibodies significantly reduce efficacy.[8,9,154] In extreme cases, the patient may show no response to injection. Therefore, several strategies have been explored to address the potential diminished potency of the toxins: 1) increasing the toxin purity, 2) reducing dosages to minimize immune response, 3) increasing treatment intervals, and 4) using a different BoNT serotype. An additional approach consists of developing BoNT/A agonists that allow lower BoNT/A dosage while maintaining efficient metalloprotease activity. A structure–activity study of 2-acylguanidine-5-phenylthiophenes toward the agonism of BoNT/A catalytic activity has unveiled a series of small molecules capable of "superactivating" LC/A with unparalleled potencies.[155] Recognizing the functional similarities between the LC/A inhibitor L-arginine hydroxamic acid and 2-acylthiophene (Zn^{2+}-binding moiety) and acylguanidine (Arg mimic), the initial intent was to develop potent LC/A inhibitors. However, the opposite phenomonen was observed. The two notable compounds **45** and **46** (Scheme 12) displayed astounding activation, with up to a 14-fold enhancement in the rate of SNAP25 cleavage (**46**).[155] Initially, protease activation by such compounds was anticipated to be due to their detergent-like properties since there are cases in the literature where such phenomena



45 46

Scheme 12. 2-Acylguanidyl-5-phenylthiophene agonists with 14-fold enhanced activation of LC/A cleavage.

occur.[156,157] However, the activation profile at drug concentrations below the critical micelle concentration (CMC) discount the possibility of a detergent-like mechanism for the action, as activation by detergents on such proteases as chymotrypsin occur solely at detergent concentrations above the CMC.[156,157]

4.2. ***BoNT-Based peptides***

An example where synthetic peptides based on BoNT have been exploited is represented by the hexapeptide acetyl-EEMQRR-NH_2.[158,159] This peptide has found use as an antiwrinkle agent, similar to the natural BoNT/A-based cosmetic botox. The peptide, referred to as argireline, was shown to act in similar fashion to BoNT/A by preventing the release of catecholamine in chromaffin cells in addition to attenuating wrinkle depth in healthy human subjects. Argireline was found to be significantly less potent than BoNT/A, however, it was much less toxic ($>2000\ mg\,kg^{-1}$) than BoNT/A ($20\ ng\,kg^{-1}$).[158]

5. Summary and Outlook

Much as Dr. Jekyll and his alter ego, Mr. Hyde, symbolically represented the duality of human nature, BoNTs also possess two seemingly disparate characteristics. The first is as a widely utilized therapeutic and cosmetic agent with an increasing number of applications, while the second is as a destructive agent of biowarfare and bioterrorism. Ultimately, both traits are a result of the ability of BoNTs to disrupt neuromuscular function by inhibition of acetylcholine release from the synaptic cleft, with advanced forms of intoxication resulting in death through cardiac arrest or respiratory failure.

Recent events, including September 11, 2001, have resulted in dramatically increased funding levels from governmental agencies for research on bioterrorism, which has led to a wealth of knowledge aimed at preventing the effects of, among others, a BoNT-based bioattack. Advances in the structure–function relationships of BoNTs as well as the biochemical mechanism of action have led to more sophisticated designs of vaccines and ushered in a new era in the development of small-molecule BoNT inhibitors. While great strides have been made in a variety of approaches, the therapeutic utility of any reported agent is inadequate for a number of reasons, including a limited window of efficacy, poor cell permeability, and short serum half-life. Thus, the recognized challenge for the future is the development of antagonists of BoNT intoxication that optimize in vivo efficacy by addressing many of these factors. To achieve these goals, increased research efforts are needed towards the validation of model systems (for example, cell-based systems[126] or bioassays) that accurately predict in vivo efficacy, including those that faithfully recapitulate the intracellular environment experienced by the LC/A metalloprotease after translocation out of endosomes. Additionally, small molecules that possess novel mechanisms of inhibition as an alternative to LC competitive inhibitors are also of significant mechanistic and clinical interest. As such, techniques such as combinatorial chemistry and high-throughput screening may enable unparalleled discoveries into the molecular mechanism of BoNT intoxication, and provide powerful research tools as well as lead molecules for the development of next-generation botulism therapeutics.

We acknowledge Naomi Sleder for assistance in the preparation of Figure 1. We thank The Skaggs Institute for Chemical Biology and the National Institutes of Health for financial support (AI66507 and AI30050).

Received: December 4, 2007

[1] E. J. Schantz, E. A. Johnson, *Microbiol. Rev.* **1992**, *56*, 80–99.
[2] B. R. Singh, *Nat. Struct. Biol.* **2000**, *7*, 617–619.
[3] J. Kerner, *Neue Beobachtungen über die in Württemberg so häufig vorfallenden tödlichen Vergiftungen durch den Genuss geräucherter Würste*, Osiander, Tübingen, **1820**.
[4] J. Kerner, *Das Fettgift oder die Fettsäure und ihre Wirkungen auf den thierischen Organismus, ein Beytrag zur Untersuchung des in verdorbenen Würsten giftig wirkenden Stoffes*, Cotta, Stuttgart, **1822**.
[5] J. G. Steinbuch, *Tueb. Bl. Naturwiss. Arzneykunde* **1817**, *3*, 26–52.
[6] J. Kerner, *Tueb. Bl. Naturwiss. Arzneykunde* **1817**, *3*, 1–25.
[7] E. P. M. Van Ermengem, *Z. Hyg. Infektionskrankh.* **1897**, *26*, 1–56.
[8] D. D. Truong, W. H. Jost, *Parkinsonism Relat. Disord.* **2006**, *12*, 331–355.
[9] R. Hackett, P. C. A. Kam, *Med. Chem.* **2007**, *3*, 333–345.
[10] S. S. Arnon, R. Schechter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow, K. Tonat, *J. Am. Med. Assoc.* **2001**, *285*, 1059–1070.
[11] S. L. Osborne, C. F. Latham, P. J. Wen, S. Cavaignac, J. Fanning, P. G. Foran, F. A. Meunier, *J. Neurosci. Res.* **2007**, *85*, 1149–1158.
[12] K. Oguma, Y. Fujinaga, K. Inoue, *Microbiol. Immunol.* **1995**, *39*, 161–168.
[13] G. Schiavo, M. Matteoli, C. Montecucco, *Physiol. Rev.* **2000**, *80*, 717–766.
[14] L. L. Simpson, *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 167–193.
[15] C. Montecucco, *Trends Biochem. Sci.* **1986**, *11*, 314–317.
[16] M. Kitamura, M. Iwamori, Y. Nagai, *Biochim. Biophys. Acta Gen. Subj.* **1980**, *628*, 328–335.
[17] M. Kitamura, K. Takamiya, S. Aizawa, K. Furukawa, K. Furukawa, *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1999**, *1441*, 1–3.
[18] A. Rummel, T. Eichner, T. Weil, T. Karnath, A. Gutcaits, S. Mahrhold, K. Sandhoff, R. L. Proia, K. R. Acharya, H. Bigalke, T. Binz, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 359–364.

[19] M. R. Baldwin, J. J. Kim, J. T. Barbieri, *Nat. Struct. Mol. Biol.* **2007**, *14*, 9 – 10.
[20] G. Matthews, *Annu. Rev. Neurosci.* **1996**, *19*, 219 – 233.
[21] W. J. Betz, J. K. Angleson, *Annu. Rev. Physiol.* **1998**, *60*, 347 – 363.
[22] M. R. Baldwin, J. T. Barbieri, *Biochemistry* **2007**, *46*, 3200 – 3210.
[23] M. Matteoli, K. Takei, M. S. Perin, T. C. Südhof, P. De Camilli, *J. Cell Biol.* **1992**, *117*, 849 – 861.
[24] S. Mahrhold, A. Rummel, H. Bigalke, B. Davletov, T. Binz, *FEBS Lett.* **2006**, *580*, 2011 – 2014.
[25] M. Dong, F. Yeh, W. H. Tepp, C. Dean, E. A. Johnson, R. Janz, E. R. Chapman, *Science* **2006**, *312*, 592 – 596.
[26] K. Sandvig in *Bacterial Protein Toxins* (Eds.: D. L. Burns, J. T. Barbieri, B. H. Iglewski), American Society for Microbiology, Washington, **2003**, pp. 157 – 172.
[27] L. L. Simpson, *J. Pharmacol. Exp. Ther.* **1982**, *222*, 43 – 48.
[28] D. H. Hoch, M. Romero-Mira, B. E. Ehrlich, A. Finkelstein, B. R. DasGupta, L. L. Simpson, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 1692 – 1696.
[29] L. K. Koriazova, M. Montal, *Nat. Struct. Biol.* **2003**, *10*, 13 – 18.
[30] A. Fischer, M. Montal, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 10447 – 10452.
[31] A. Fischer, M. Montal, *J. Biol. Chem.* **2007**, *282*, 29604 – 29611.
[32] Y. Humeau, F. Doussau, N. J. Grant, B. Poulain, *Biochimie* **2000**, *82*, 427 – 446.
[33] F. A. Meunier, G. Schiavo, J. Molgó, *J. Physiol.* **2002**, *96*, 105 – 113.
[34] G. Schiavo, F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B. R. DasGupta, C. Montecucco, *Nature* **1992**, *359*, 832 – 835.
[35] S. Chen, J. T. Barbieri, *J. Biol. Chem.* **2006**, *281*, 10906 – 10911.
[36] S. Chen, J. T. Barbieri, *J. Biol. Chem.* **2007**, *282*, 25540 – 25547.
[37] S. Chen, J. J. Kim, J. T. Barbieri, *J. Biol. Chem.* **2007**, *282*, 9621 – 9627.
[38] M. A. Fiock, M. A. Cardella, N. F. Gearinger, *J. Immunol.* **1963**, *90*, 697 – 702.
[39] L. S. Siegel, *J. Clin. Microbiol.* **1988**, *26*, 2351 – 2356.
[40] L. S. Siegel, *J. Clin. Microbiol.* **1989**, *27*, 1906 – 1908.
[41] M. P. Byrne, L. A. Smith, *Biochimie* **2000**, *82*, 955 – 966.
[42] C. L. Pier, W. H. Tepp, M. Bradshaw, E. A. Johnson, J. T. Barbieri, M. R. Baldwin, *Infect. Immun.* **2008**, *76*, 437 – 442.
[43] N. F. Fairweather, V. A. Lyness, D. J. Maskell, *Infect. Immun.* **1987**, *55*, 2541 – 2545.
[44] D. B. Lacy, R. C. Stevens, *J. Mol. Biol.* **1999**, *291*, 1091 – 1104.
[45] D. B. Lacy, W. Tepp, A. C. Cohen, B. R. DasGupta, R. C. Stevens, *Nat. Struct. Biol.* **1998**, *5*, 898 – 902.
[46] C. Montecucco, G. Schiavo, *Q. Rev. Biophys.* **1995**, *28*, 423 – 472.
[47] D. E. Thompson, J. K. Brehm, J. D. Oultram, T. J. Swinfield, C. C. Shone, T. Atkinson, J. Melling, N. P. Minton, *Eur. J. Biochem.* **1990**, *189*, 73 – 81.
[48] H. F. LaPenotiere, M. A. Clayton, J. L. Middlebrook, *Toxicon* **1995**, *33*, 1383 – 1386.
[49] M. A. Clayton, J. M. Clayton, D. R. Brown, J. L. Middlebrook, *Infect. Immun.* **1995**, *63*, 2738 – 2742.
[50] L. A. Smith, *Toxicon* **1998**, *36*, 1539 – 1548.
[51] M. P. Byrne, T. J. Smith, V. A. Montgomery, L. A. Smith, *Infect. Immun.* **1998**, *66*, 4817 – 4822.
[52] K. J. Potter, M. A. Bevins, E. V. Vassilieva, V. R. Chiruvolu, T. Smith, L. A. Smith, M. M. Meagher, *Protein Expression Purif.* **1998**, *13*, 357 – 365.
[53] K. J. Potter, W. Zhang, L. A. Smith, M. M. Meagher, *Protein Expression Purif.* **2000**, *19*, 393 – 402.
[54] M. R. Baldwin, W. H. Tepp, C. L. Pier, M. Bradshaw, M. Ho, B. A. Wilson, R. B. Fritz, E. A. Johnson, J. T. Barbieri, *Infect. Immun.* **2005**, *73*, 6998 – 7005.
[55] M. P. Dux, R. Barent, J. Sinha, M. Gouthro, T. Swanson, A. Barthuli, M. Inan, J. T. Ross, L. A. Smith, T. J. Smith, R. Webb, B. Loveless, I. Henderson, M. M. Meagher, *Protein Expression Purif.* **2006**, *45*, 359 – 367.
[56] J. C. Lee, H. J. Hwang, Y. Sakaguchi, Y. Yamamoto, H. Arimitsu, T. Tsuji, T. Watanabe, T. Ohyama, T. Tsuchiya, K. Oguma, *Microbiol. Immunol.* **2007**, *51*, 445 – 455.
[57] R. P. Webb, T. J. Smith, P. M. Wright, V. A. Montgomery, M. M. Meagher, L. A. Smith, *Vaccine* **2007**, *25*, 4273 – 4282.
[58] J. Boles, M. West, V. Montgomery, R. Tammariello, M. L. Pitt, P. Gibbs, L. Smith, R. D. LeClaire, *Toxicon* **2006**, *47*, 877 – 884.
[59] P. Pushko, M. Parker, G. V. Ludwig, N. L. Davis, R. E. Johnston, J. F. Smith, *Virology* **1997**, *239*, 389 – 401.
[60] J. L. Middlebrook, *Adv. Drug Delivery Rev.* **2005**, *57*, 1415 – 1423.
[61] M. Zeng, Q. Xu, M. Elias, M. E. Pichichero, L. L. Simpson, L. A. Smith, *Vaccine* **2007**, *25*, 7540 – 7548.
[62] J. S. Lee, P. Pushko, M. D. Parker, M. T. Dertzbaugh, L. A. Smith, J. F. Smith, *Infect. Immun.* **2001**, *69*, 5709 – 5715.
[63] R. H. Shyu, M. F. Shaio, S. S. Tang, H. F. Shyu, C. F. Lee, M. H. Tsai, J. E. Smith, H. H. Huang, J. J. Wey, J. L. Huang, H. H. Chang, *J. Biomed. Sci.* **200**, *7*, 51 – 57.
[64] A. P. Jathoul, J. L. Holley, H. S. Garmory, *Vaccine* **2004**, *22*, 3942 – 3946.
[65] J. Clayton, J. L. Middlebrook, *Vaccine* **2000**, *18*, 1855 – 1862.
[66] A. Casadevall, *Emerging Infect. Dis.* **2002**, *8*, 833 – 841.
[67] C. O. Tacket, W. X. Shandera, J. M. Mann, N. T. Hargrett, P. A. Blake, *Am. J. Med.* **1984**, *76*, 794 – 798.
[68] R. E. Black, R. A. Gunn, *Am. J. Med.* **1980**, *69*, 567 – 570.
[69] S. S. Arnon, R. Schechter, S. E. Maslanka, N. P. Jewell, C. L. Hatheway, *N. Engl. J. Med.* **2006**, *354*, 462 – 471.
[70] A. Norrby-Teglund, H. Basma, J. Anderson, A. McGeer, D. E. Low, M. Kotb, *Clin. Infect. Dis.* **1998**, *26*, 631 – 638.
[71] A. Farrugia, P. Poulis, *Prog. Transfus. Med.* **2001**, *11*, 63 – 74.
[72] H. B. Slade, *Clin. Diagn. Lab. Immunol.* **1994**, *1*, 613 – 619.
[73] P. T. Jones, P. H. Dear, J. Foote, M. S. Neuberger, G. Winter, *Nature* **1986**, *321*, 522 – 525.
[74] J. D. Marks, *Mov. Disord.* **2004**, *19*, S101 – S108.
[75] S. Kozaki, Y. Kamata, T. Nagai, J. Ogasawara, G. Sakaguchi, *Infect. Immun.* **1986**, *52*, 786 – 791.
[76] S. Bavari, D. D. Pless, E. R. Torres, F. J. Lebeda, M. A. Olson, *Vaccine* **1998**, *16*, 1850 – 1856.
[77] D. D. Pless, E. R. Torres, E. K. Reinke, S. Bavari, *Infect. Immun.* **2001**, *69*, 570 – 574.
[78] G. H. Yang, K. S. Kim, H. W. Kim, S. T. Jeong, G. H. Huh, J. C. Kim, H. H. Jung, *Toxicon* **2004**, *44*, 19 – 25.
[79] P. Amersdorfer, C. Wong, S. Chen, T. Smith, S. Deshpande, R. Sherican, R. Finnern, J. D. Marks, *Infect. Immun.* **1997**, *65*, 3743 – 3752.
[80] P. Amersdorfer, C. Wong, T. Smith, S. Chen, S. Deshpande, R. Sheridan, J. D. Marks, *Vaccine* **2002**, *20*, 1640 – 1648.
[81] D. C. Mah, W. G. Hu, J. K. Pon, S. A. Masri, R. E. Fulton, P. L. Monette, L. P. Nagata, *Hybridoma Hybridomics* **2003**, *22*, 277 – 283.
[82] B. Hallis, S. Fooks, C. Shone, P. Hambleton in *Botulinum and tetanus neurotoxins: neurotransmission and biomedical aspects* (Ed.: B. R. DasGupta), **1993**, Plenum, New York, pp. 433 – 436.
[83] A. Nowakowski, C. Wang, D. B. Powers, P. Amersdorfer, T. J. Smith, V. A. Montgomery, R. Sheridan, R. Blake, L. A. Smith, J. D. Marks, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11346 – 11350.
[84] L. L. Simpson, M. M. Rapport, *J. Neurochem.* **1971**, *18*, 1751 – 1759.
[85] L. L. Simpson, M. M. Rapport, *J. Neurochem.* **1971**, *18*, 1341 – 1343.
[86] Y. Kamata, M. Yoshimoto, S. Kozaki, *Toxicon* **1997**, *35*, 1337 – 1340.

[87] R. R. Kale, C. M. Clancy, R. M. Vermillion, E. A. Johnson, S. S. Iyer, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2459 – 2464.
[88] N. Bakry, Y. Kamata, L. L. Simpson, *J. Pharmacol. Exp. Ther.* **1991**, *258*, 830 – 836.
[89] L. L. Simpson, *J. Pharmacol. Exp. Ther.* **1983**, *225*, 546 – 552.
[90] L. L. Simpson, J. A. Coffield, N. Bakry, *J. Pharmacol. Exp. Ther.* **1994**, *269*, 256 – 262.
[91] R. E. Sheridan, *Toxicon* **1996**, *34*, 849 – 855.
[92] J. E. Keller, F. Cai, E. A. Neale, *Biochemistry* **2004**, *43*, 526 – 532.
[93] S. S. Deshpande, R. E. Sheridan, M. Adler, *Toxicon* **1997**, *35*, 433 – 445.
[94] M. Adler, R. E. Dinterman, R. W. Wannemacher, *Toxicon* **1997**, *35*, 1089 – 1100.
[95] T. Söllner, S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, J. E. Rothman, *Nature* **1993**, *362*, 318 – 324.
[96] O. Rossetto, G. Schiavo, C. Montecucco, B. Poulain, F. Deloye, L. Lozzi, C. C. Shone, *Nature* **1994**, *372*, 415 – 416.
[97] J. Hayden, J. Pires, M. Hamilton, G. Moore, *Proc. West. Pharmacol. Soc.* **2000**, *43*, 71 – 74.
[98] J. Hayden, J. Pires, S. Roy, M. Hamilton, G. J. Moore, *J. Appl. Toxicol.* **2003**, *23*, 1 – 7.
[99] G. J. Moore, D. M. Moore, S. S. Roy, L. J. Hayden, M. G. Hamilton, N. W. C. Chan, W. E. Lee, *Mol. Diversity* **2006**, *10*, 9 – 16.
[100] J. J. Schmidt, R. G. Stafford, K. A. Bostian, *FEBS Lett.* **1998**, *435*, 61 – 64.
[101] J. J. Schmidt, R. G. Stafford, *FEBS Lett.* **2002**, *532*, 423 – 426.
[102] M. Brewer, C. A. James, D. H. Rich, *Org. Lett.* **2004**, *6*, 4779 – 4782.
[103] B. E. Haug, D. H. Rich, *Org. Lett.* **2004**, *6*, 4783 – 4786.
[104] C. Sukonpan, T. Oost, M. Goodnough, W. Tepp, E. A. Johnson, D. H. Rich, *J. Pept. Res.* **2004**, *63*, 181 – 193.
[105] T. Oost, C. Sukonpan, M. Brewer, M. Goodnough, W. Tepp, E. A. Johnson, D. H. Rich, *Biopolymers* **2003**, *71*, 602 – 619.
[106] C. Anne, S. Turcaud, J. Quancard, F. Teffo, H. Meudal, M. C. Fournié-Zaluski, B. P. Roques, *J. Med. Chem.* **2003**, *46*, 4648 – 4656.
[107] L. Martin, F. Cornille, S. Turcaud, H. Meudal, B. P. Roques, M. C. Fournié-Zaluski, *J. Med. Chem.* **1999**, *42*, 515 – 525.
[108] L. Martin, F. Cornille, P. Coric, B. P. Roques, M. C. Fournié-Zaluski, *J. Med. Chem.* **1998**, *41*, 3450 – 3460.
[109] M. A. Hanson, R. C. Stevens, *Nat. Struct. Biol.* **2000**, *7*, 687 – 692.
[110] S. Swaminathan, S. Eswaramoorthy, *Nat. Struct. Biol.* **2000**, *7*, 693 – 699.
[111] A. Blommaert, S. Turcaud, C. Anne, B. P. Roques, *Bioorg. Med. Chem.* **2004**, *12*, 3055 – 3062.
[112] E. R. Evans, J. M. Sutton, A. Gravett, C. C. Shone, *Toxicon* **2005**, *46*, 446 – 453.
[113] A. G. Zdanovsky, N. V. Karassina, D. Simpson, M. V. Zdanovskaia, *J. Protein Chem.* **2001**, *20*, 73 – 80.
[114] K. P. A. B. Yiadom, S. Muhie, D. C. H. Yang, *Biochem. Biophys. Res. Commun.* **2005**, *335*, 1247 – 1253.
[115] G. E. Boldt, J. P. Kennedy, M. S. Hixon, L. A. McAllister, J. T. Barbieri, S. Tzipori, K. D. Janda, *J. Comb. Chem.* **2006**, *8*, 513 – 521.
[116] Z. Fu, S. Chen, M. R. Baldwin, G. E. Boldt, A. Crawford, K. D. Janda, J. T. Barbieri, J. J. Kim, *Biochemistry* **2006**, *45*, 8903 – 8911.
[117] G. E. Boldt, J. P. Kennedy, K. D. Janda, *Org. Lett.* **2006**, *8*, 1729 – 1732.
[118] J. G. Park, P. C. Sill, E. F. Makiyi, A. T. Garcia-Sosa, C. B. Millard, J. J. Schmidt, Y. P. Pang, *Bioorg. Med. Chem.* **2006**, *14*, 395 – 408.
[119] J. C. Burnett, J. J. Schmidt, R. G. Stafford, R. G. Panchal, T. L. Nguyen, A. R. Hermone, J. L. Vennerstrom, C. F. McGrath, D. J. Lane, E. A. Sausville, D. W. Zaharevitz, R. Gussio, S. Bavari, *Biochem. Biophys. Res. Commun.* **2003**, *310*, 84 – 93.
[120] N. R. Silvaggi, G. E. Boldt, M. S. Hixon, J. P. Kennedy, S. Tzipori, K. D. Janda, K. N. Allen, *Chem. Biol.* **2007**, *14*, 533 – 542.
[121] R. E. Sheridan, S. S. Deshpande, *Toxicon* **1995**, *33*, 539 – 549.
[122] L. L. Simpson, J. A. Coffield, N. Bakry, *J. Pharmacol. Exp. Ther.* **1993**, *267*, 720 – 727.
[123] F. Cai, C. B. Adrion, J. E. Keller, *Infect. Immun.* **2006**, *74*, 5617 – 5624.
[124] F. N. Fu, R. B. Lomneth, S. Cai, B. R. Singh, *Biochemistry* **1998**, *37*, 5267 – 5278.
[125] L. Li, B. R. Singh, *Biochemistry* **2000**, *39*, 10581 – 10586.
[126] L. M. Eubanks, M. S. Hixon, W. Jin, S. Hong, C. M. Clancy, W. H. Tepp, M. R. Baldwin, C. J. Malizio, M. C. Goodnough, J. T. Barbieri, E. A. Johnson, D. L. Boger, T. J. Dickerson, K. D. Janda, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2602 – 2607.
[127] G. E. Boldt, L. M. Eubanks, K. D. Janda, *Chem. Commun.* **2006**, 3063 – 3065.
[128] M. A. Hanson, T. K. Oost, C. Sukonpan, D. H. Rich, R. C. Stevens, *J. Am. Chem. Soc.* **2000**, *122*, 11268 – 11269.
[129] M. Adler, J. D. Nicholson, B. E. Hackley, Jr., *FEBS Lett.* **1998**, *429*, 234 – 238.
[130] R. R. Roe, Y. P. Pang, *J. Mol. Model.* **1999**, *5*, 134 – 140.
[131] J. Tang, J. G. Park, C. B. Millard, J. J. Schmidt, Y. P. Pang, *PLoS One* **2007**, *2*, e761.
[132] J. C. Burnett, G. Ruthel, C. M. Stegmann, R. G. Panchal, T. L. Nguyen, A. R. Hermone, R. G. Stafford, D. J. Lane, T. A. Kenny, C. F. McGrath, P. Wipf, A. M. Stahl, J. J. Schmidt, R. Gussio, A. T. Brunger, S. Bavari, *J. Biol. Chem.* **2006**, *282*, 5004 – 5014.
[133] B. Segelke, M. Knapp, S. Kadkhodayan, R. Balhorn, B. Rupp, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 6888 – 6893.
[134] J. C. Burnett, D. Opsenica, K. Sriraghavan, R. G. Panchal, G. Ruthel, A. R. Hermone, T. L. Nguyen, T. A. Kenny, D. J. Lane, C. F. McGrath, J. J. Schmidt, J. L. Vennerstrom, R. Gussio, B. A. Solaja, S. Bavari, *J. Med. Chem.* **2007**, *50*, 2127 – 2136.
[135] B. Rudy, *Neuroscience* **1988**, *25*, 729 – 749.
[136] H. Lundh, S. G. Cull-Candy, S. Leander, S. Thesleff, *Brain Res.* **1976**, *110*, 194 – 198.
[137] H. Lundh, S. Leander, S. Thesleff, *J. Neurol. Sci.* **1977**, *32*, 29 – 43.
[138] P. Metezeau, M. Desban, *Toxicon* **1982**, *20*, 649 – 654.
[139] M. Adler, J. Scovill, G. Parker, F. J. Lebeda, J. Piotrowski, S. S. Deshpande, *Toxicon* **1995**, *33*, 527 – 537.
[140] M. Adler, D. A. Macdonald, L. C. Sellin, G. W. Parker, *Toxicon* **1996**, *34*, 237 – 249.
[141] M. Adler, B. Capacio, S. S. Deshpande, *Toxicon* **2000**, *38*, 1381 – 1388.
[142] E. Satoh, T. Ishii, Y. Shimizu, S. Sawamura, M. Nishimura, *Br. J. Pharmacol.* **2001**, *132*, 797 – 798.
[143] E. Satoh, T. Ishii, Y. Shimizu, S. Sawamura, M. Nishimura, *Pharmacol. Toxicol.* **2002**, *90*, 199 – 202.
[144] M. Nakatani, *Heterocycles* **1999**, *50*, 595 – 609.
[145] Y. S. Xie, P. G. Fields, M. B. Isman, W. K. Chen, X. Zhang, *J. Stored Prod. Res.* **1995**, *31*, 259 – 265.
[146] J. B. Zhou, Y. Minami, F. Yagi, K. Tadera, M. Nakatani, *Heterocycles* **1997**, *45*, 1781 – 1786.
[147] Y. L. Shi, M. F. Li, *Prog. Neurobiol.* **2007**, *82*, 1 – 10.
[148] Y. L. Shih, N. S. Wei, Y. Q. Yang, Z. X. Wang, *Acta Physiol. Sin.* **1980**, *32*, 293 – 297.
[149] Y. Shih, K. Hsu, *Jpn. J. Physiol.* **1983**, *33*, 677 – 680.
[150] M. F. Li, Y. Wu, Z. F. Wang, Y. L. Shi, *Neurosci. Res.* **2004**, *49*, 197 – 203.
[151] Z. F. Wang, Y. L. Shi, *Neuroscience* **2001**, *104*, 41 – 47.

[152] Z. F. Wang, Y. L. Shi, *Neurosci. Res.* **2001**, *40*, 211 – 215.
[153] M. Z. Tang, Z. F. Wang, Y. L. Shi, *Neurosci. Res.* **2003**, *45*, 225 – 231.
[154] E. A. Johnson, *Annu. Rev. Microbiol.* **1999**, *53*, 551 – 575.
[155] L. A. McAllister, M. S. Hixon, J. P. Kennedy, T. J. Dickerson, K. D. Janda, *J. Am. Chem. Soc.* **2006**, *128*, 4176 – 4177.
[156] P. Viparelli, F. Alfani, M. Cantarella, *Biochem. J.* **1999**, *344*, 765 – 773.
[157] M. S. Celej, M. G. D'Andrea, P. T. Campana, G. D. Fidelio, M. L. Bianconi, *Biochem. J.* **2004**, *378*, 1059 – 1066.
[158] C. Blanes-Mira, J. Clemente, G. Jodas, A. Gil, G. Fernández-Ballester, B. Ponsati, L. Gutierrez, E. Pérez-Payá, A. Ferrer-Montiel, *Int. J. Cosmet. Sci.* **2002**, *24*, 303 – 310.
[159] M. A. Ruiz, B. Clares, M. E. Morales, S. Cazalla, V. Gallardo, *J. Cosmet. Sci.* **2007**, *58*, 157 – 171.