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Scorpion toxins for the reversal of BoNT-induced paralysis

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Abstract

The botulinum neurotoxins, characterized by their neuromuscular paralytic effects, are the most toxic proteins known to man. Due to their extreme potency, ease of production, and duration of activity, the BoNT proteins have been classified by the Centers for Disease Control as high threat agents for bioterrorism. In an attempt to discover effective BoNT therapeutics, we have pursued a strategy in which we leverage the blockade of K⁺ channels that ultimately results in the reversal of neuromuscular paralysis. Towards this end, we utilized peptides derived from scorpion venom that are highly potent K⁺ channel blockers. Herein, we report the synthesis of charybdotoxin, a 37 amino acid peptide, and detail its activity, along with iberiotoxin and margatoxin, in a mouse phrenic nerve hemidiaphragm assay in the absence and the presence of BoNT/A.

Keywords

Botulinum neurotoxin; Scorpion toxin; Bioterrorism; Aminopyridine; Phrenic nerve hemidiaphragm

Among the deadliest toxins in the world are the botulinum neurotoxins (BoNTs), produced by the Gram positive bacterium *Clostridium botulinum*, with lethal doses as low as 1 ng/kg body weight. These toxins, which are the etiological agents underlying the human disease botulism, block peripheral neuromuscular transmission causes the characteristic flaccid paralysis. This paralytic activity eventually affects respiratory functions, which can prove fatal in the absence of expedient intensive medical care and artificial respiration. It is this extreme potency, coupled with the protracted duration of paralysis and relative ease of production that has lead the U.S. Centers for Disease Control and Prevention to classify BoNT as a category A bioterrorism agent; that is, a substance with high mortality rates that may be efficiently publicly dispersed.¹ Furthermore, options for supportive care and

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at

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treatment of intoxication are limited, and as such we have sought to develop approaches targeted at both symptomatic relief and reversal of paralysis caused by the most potent of the seven BoNT serotypes, serotype A (BoNT/A).

Cellular paralysis caused by BoNT occurs in a four-step process beginning with binding of the toxin to cellular receptor proteins. This is followed by receptor mediated endocytosis and transport of the light chain domain (LC). Finally, cleavage of one of three SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins by the LC metalloprotease results in the loss of acetylcholine release at the neuromuscular junction, thereby eliminating neurotransmission.² Specifically, BoNT/A cleaves the SNAP-25 protein to prevent neurotransmitter release. In the development of BoNT therapeutics, this progression of paralysis has traditionally been targeted from three avenues: (1) antagonism of toxin binding to neuronal cell surface, (2) prevention of LC transport in to the cytosol, and (3) inhibition of the proteolytic activity of the LC.³ However, an additional approach has been introduced for the treatment of BoNT/A that does not target the toxin itself but instead is aimed at the reversal of cellular paralysis.

Medical countermeasures for BoNT intoxication rely on pre-exposure vaccination or post-exposure administration of antitoxin and intensive care often involving intubation and mechanical respiration. As such, a large-scale release of BoNT would necessitate more efficient intervention strategies to restore neuromuscular activity. One such potential avenue lies in the blockade of K^+ channels, as the elimination of K^+ currents results in elevated Ca^{2+} influx, which, in turn, promotes acetylcholine release within the synaptic cleft. This approach has been successfully demonstrated with the aminopyridines, a class of K^+ channel blockers that are effective in the reversal of BoNT/A induced paralysis in phrenic nerve-hemidiaphragm preparations and in the rescue of BoNT/A-poisoned mice in lethality assays.⁴⁻⁶ However, these compounds can also produce toxic side effects largely due to blood brain barrier (BBB) penetration. Abnormal muscle contractions have also been observed with aminopyridine concentrations needed to fully restore BoNT/A induced paralysis.⁷ Nevertheless, based on the effectiveness of K^+ channel blockers we hypothesized that a series of scorpion toxins, which are potent K^+ channel blockers, might serve to restore proper nerve function in BoNT/A intoxicated cells while minimizing the side effects caused by BBB penetration of the aminopyridines.

Neurotoxic peptides isolated from various venomous animals, such as scorpions, are extremely specific and potent K^+ channel blockers.^{8, 9} As such, these toxins have become powerful biochemical tools for the study of K^+ channels, and have been used to identify and characterize K^+ channels.⁹ Additionally, radio- and fluorescently-labeled analogs have been utilized for channel localization studies, and a number of scorpion toxins are under investigation for molecular therapeutic applications against diseases such as cardiac arrhythmia, hypertension, multiple sclerosis, and other autoimmune diseases.^{10, 11} These peptides also exhibit low BBB penetrability in adult animals,¹² which may help to minimize certain side effects associated with the administration of the aminopyridines. Interestingly, recent studies using latrotoxin, a toxin that is a component of black widow spider venom, has been demonstrated to promote calcium influx and restore neuronal activity, as well as SNAP-25 expression in BoNT/A-intoxicated neurons, although neurotoxicity considerations may hinder its therapeutic development.¹³ Additionally, crude venom isolated from the scorpion *Androctonus australis* is effective in reversing BoNT-induced paralysis in frog nerve-muscle preparations.¹⁴ As such, these findings advocate the value of venom components for the treatment of BoNT/A.

In contrast to the aminopyridines, which penetrate the cellular membrane and obstruct the intracellular surface of the K^+ channels,^{15, 16} scorpion toxin peptides bind at the

extracellular surface of the channel.¹⁷ Because these two classes of K⁺ channel blockers possess different binding modes, they may be able to complement each other to produce a more effective blockade of the K⁺ channels and ultimately symptomatic relief of BoNT-inflicted muscle paralysis. Herein, we report our findings that the scorpion toxins, only when used in conjunction with 3–4 diaminopyridine (3,4-DAP), heightened muscle contraction in the mouse phrenic nerve hemidiaphragm assay. However, when used alone the scorpion toxins do not provide enhanced muscle contractions in this assay, nor are they effective in restoring neuromuscular function in BoNT/A intoxicated preparations.

While it is known that the blockade of K⁺ channels is effective the reversal of BoNT-induced paralysis, the individual K⁺ channels underlying this activity remain enigmatic. As such, we selected three representative scorpion toxins to probe the contributions of two families of K⁺ channels: voltage-gated channels and calcium-activated channels. We chose voltage-gated channels due to their sensitivity to aminopyridine blockade, and Ca²⁺-activated channels based on reports that obstruction by iberiotoxin results in increased neurotransmitter release.^{18, 19} Thus, we hypothesized that these specific K⁺ channels would be appropriate targets for the restoration of neurotransmission in BoNT-poisoned cells. Our initial focus was on the following compounds with selectivity towards specific K⁺ channel subtypes: margatoxin (K_v1.3-selective, ID₅₀ = 50 pM),²⁰ iberiotoxin (BK K_{Ca}1.1-selective, ID₅₀ = 250 pM),²¹ and charybdotoxin (potent non-selective antagonist of K_v1.1–1.3, 1.6, BK K_{Ca}1.1 and IK K_{Ca}3.1 channels, ID₅₀ = 25 pM).^{22–27} We also reasoned that the high potency of these toxins would also allow the treatment of BoNT-induced paralysis at concentrations well below their LD₅₀ (e.g. ChTX = 8 ng/g and MgTX = 6 ng/g) mouse models.²⁸

The scorpion toxins feature significant sequence homology, and an overall positive charge, owing to the high abundance of basic amino acids, contributes to their K⁺ channel blocking activity. This activity also relies on a “functional dyad” consisting of a lysine residue that essentially blocks the pore of the channel, and a proximal aromatic residue (e.g. tyrosine or phenylalanine) and also feature the Cysteine-Stabilized α/β motif (CS- $\alpha\beta$), in which an α -helix is linked to one strand of a β -sheet structure by two disulfide bridges, C_i-C_j and C_{i+4}-C_{j+2}.^{9,29–31} In the case of charybdotoxin (ChTX – C⁷-C²⁸, C¹³-C³³, C¹⁷-C³⁵), margatoxin (MgTX – C⁷-C², C¹³-C³⁴, C¹⁷-C³⁶), and iberiotoxin (IbTX – C⁷-C²⁸, C¹³-C³³, C¹⁷-C³⁵), there are three disulfide bonds that dictate the secondary structures of each peptide. This structural complexity renders synthesis difficult and previous studies on the synthesis of ChTX have reported overall yields ranging from 2 – 10%.^{32–34}

We undertook the solid phase synthesis of ChTX with the aim of establishing a reliable synthetic protocol which may be applied to large-scale toxin synthesis and the potential future construction of analogs, as well as improving the yield of the final oxidation reaction. An automated, solid-phase Fmoc synthetic protocol provided the linear peptide in 14% yield, positioning us to explore the oxidation reaction to provide the final product. Typically, air oxidation and glutathione-catalyzed oxidations have been employed in the construction of the appropriate disulfide bonds of ChTX, with overall yields ranging from 2–10%.^{32–35} In an attempt to improve upon these previous studies, we envisioned the use of the CLEAR-OX resin to promote more efficient folding of the linear peptide (Figure 2).³⁶ CLEAR-OX is a combination of the Cross-Linked Ethoxylate Acrylate Resin (CLEAR) and Ellman's reagent, which is traditionally used to detect free thiols in solution, but when attached to the resin promotes disulfide formation.³⁷ This resin has demonstrated improved yields over air oxidation techniques for the formation of disulfide bonds in peptides such as the conotoxins³⁸ and hepcidin.³⁹ However, in our hands the yield of the oxidative folding of ChTX was approximately 7-fold greater under air oxidation conditions (pH 8, 5% yield)³³ than in the presence of CLEAR-OX (0.75% yield). Furthermore, the oxidation in the presence of the resin was sluggish, as a reaction time of 24 h was required, as opposed to 8 h

under the slightly basic conditions. This observation may be due in part to the fact that CLEAR-OX is more efficient at higher peptide concentrations (6–7 mg/ml), whereas these conditions were hindered by the low solubility of the linear peptide (~ 1 mg/ml) under the prescribed conditions.³⁸

With a synthetic protocol in hand to obtain ChTX, the three toxins (ChTX, MgTX, IbTX) were evaluated in mouse phrenic nerve hemidiaphragm assays to determine their capacity to induce muscle tension and restore neurotransmission in BoNT intoxicated cells. In these assays, ChTX and IbTX produced a small increase in tension at 10 nM, but exhibited little additional twitch potentiation at 30 and 100 nM (Figure 3). As such, it is likely that there is a small population of sensitive channels that are inhibited at 10 nM. However, these channels are likely a small component of the K⁺ channel population, and their inhibition does not lead to the level of potentiation observed with the aminopyridines.^{3–6} Although the toxins were unable to potentiate muscle twitch tension on their own, in combination with 3,4-DAP the toxins did increase muscle twitch tension (Figure 4). We attribute this to the fact that after 3,4-DAP treatment, the end-plate potentials are markedly potentiated based on the increase tension observed. There are also presumably muscle fibers that are below threshold which may be elevated to threshold by the scorpion toxins, since it now requires a smaller boost, accounting for the increase in activity under treatment with both 3,4-DAP and scorpion toxin. However, in BoNT-intoxicated muscles that have been paralyzed by > 30%, treatment with the three scorpion toxins did not result in increased in muscle tension at any concentration tested (10–100 nM).

The variable activity of the over 100 known scorpion toxin peptides towards different subtypes of K⁺ channels,⁹ which makes these peptides invaluable tools in validating specific types of K⁺ channels present on the motor nerve terminal, is also likely the underlying reason for the inability of ChTX, MgTX, and IbTX to promote muscle tension and restore neurotransmission in BoNT intoxicated cells. This is in contrast to findings with aminopyridines, which are less potent K⁺ channel blockers than the scorpion toxins with IC₅₀ values in the micromolar range.⁴⁰ However, it may be the highly selective nature of the scorpion toxins that results in the blockade of an insufficient number of K⁺ channels to induce acetylcholine release. For example, ChTX is known to target the K_v1 family of voltage gated K⁺ channels as well as BK and IK Ca²⁺-activated channels, whereas the aminopyridines are broad spectrum K⁺ channel blockers.⁴⁰ Interestingly, previous reports using twitch tension and electrophysiological recording demonstrate that blockade of K⁺ channels in mouse triangularis sterni preparations was effective in increasing acetylcholine release in a channel-specific manner.³⁷ In this study, ITX and ChTX were effective in promoting acetylcholine release, but no augmentation was observed with the voltage-gated channel specific noxiustoxin, thus highlighting the significance of K⁺ channel selectivity to achieve the desired activity. Thus, it may be that the channels targeted by the scorpion toxins in the current study are suboptimal for the restoration of nerve activity following BoNT/A intoxication.

On the other hand, the aminopyridines, which did serve to increase twitch tension and are known to be effective in reversing BoNT/A induced paralysis, suffer from efficacy issues likely owing to their indiscriminate binding and poor affinity. Furthermore, the precise K⁺ channels responsible for the observed activity of the aminopyridines remain unknown. As such, we hypothesize that delineation of these K⁺ channels will provide the opportunity for the development of therapeutics with both increased potency and improved toxicity profiles.

In conclusion, we have demonstrated that three scorpion toxins, although known to be potent K⁺ channel blockers, are ineffective for restoring neurotransmission to cells paralyzed by BoNT/A, an unanticipated finding in light of the activity of the weaker K⁺ channel blocking

aminopyridines. Nevertheless, we will continue our efforts towards the discovery of selective, potent K⁺ channel blockers for BoNT treatment, which will require the discovery and judicious selection of targeted channels as the foundation moving forward.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures and target K⁺ channels of the scorpion toxin peptides used in this study.

Figure 2.
Structure of CLEAR-OXTM.

Figure 3.
Effect of the indicated scorpion toxins on twitch tension in control hemidiaphragm muscle.
Toxin addition was cumulative.

Figure 4. Effect of the indicated scorpion toxins (100 nM) on twitch tension in control hemidiaphragm muscle in combination with 10 μ M 3,4-DAP. Toxin addition was cumulative.