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A Cross-over Inhibitor of the Botulinum Neurotoxin Light Chain B: A Natural Product Implicating an Exosite Mechanism of Action

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Abstract

Clostridium botulinum produces the most lethal toxins known to man, as such they are high risk terrorist threats, and alarmingly there is no approved therapeutic. We report the first cross-over small molecule inhibitor of these neurotoxins and propose a mechanism by which it may impart its inhibitory activity.

Botulinum neurotoxins (BoNTs) are produced and secreted by the organisms *C. botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinense*, and are among the most toxic natural substances known to man. These neurotoxins are classified by the Centers for Disease Control and Prevention (CDC) as Category A agents, with a human 50% lethal dose (LD₅₀) of approximately 1 ng/kg of body weight for the BoNT serotype A (BoNT/A).¹ The extreme potency of the toxin coupled with its relative ease of production has raised concerns by global anti-terrorism experts as to the potential destructive uses of the neurotoxin. Therefore, there is a great need for research into the development of counter measures against these lethal toxins.

Clostridial neurotoxins including BoNT are a unique architectural class of proteins. BoNTs are comprised of a 150 kDa heavy chain (HC) domain and a 50 kDa light chain (LC) domain. The HC consists of both the binding and translocation domains responsible for the neurospecific binding, uptake and translocation of the toxin LC into the cytosol; the LC is a zinc mediated endopeptidase that cleaves specifically one of three soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins resulting in neurotransmitter blockage, paralysis and in severe cases, death.² There are seven BoNT serotypes, A–G, classified according to their intracellular SNARE protein target and site of

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cleavage. Serotypes A, B, and E are the most common agents responsible for human botulism.¹

Currently, no small molecule therapeutics are approved for the treatment of botulinum intoxication caused by any of the serotypes. Antibody based antitoxins are available, however this treatment option is ineffectual once cellular intoxication has occurred. Recent approaches for small molecule therapeutic intervention include restoration of neuromuscular transmission, blockade of LC translocation into the neuronal cell, and inhibition of the enzymatic cleavage of the target SNARE proteins by the LC.³ To date, research has primarily focused on developing inhibitors against the BoNT/A LC (LC/A) protease, due to this serotype's persistence and highly toxic nature.

Common to all botulinum serotypes is the LC domain, a Zn²⁺-dependent metalloprotease. A logical strategy for the development of inhibitors against this enzyme has been the design of small molecules containing a warhead able to coordinate the active site zinc cation, thereby inactivating the enzyme's catalytic machinery. Our laboratory has exploited this approach and has reported several of the most potent inhibitors against LC/A to date.^{4–6} However, we note that while success has been achieved using this strategy it may be susceptible to off-target toxicity, as the highly conserved Zn²⁺ binding motif is also present within the active sites of many human metalloproteases.

The BoNT/B serotype targets the SNARE protein VAMP-2 (vesicle-associated membrane protein 2), also known as synaptobrevin 2, and is both the second most toxic and common cause of human botulism. Yet, BoNT/B has received considerably less attention compared to BoNT/A, especially toward developing therapeutic small molecules for treatment of botulinum intoxication. Thus, while a small series of peptides with K_i values in the nanomolar range have been reported^{7–11}, to our knowledge there have only been two reports of small molecule inhibitors against BoNT/B and these exhibit modest inhibition constants.^{12–13} Given the dearth of small molecule inhibitors and the difficulties of developing peptides as drug candidates, there is a need for research in the development of BoNT/B small molecule therapeutics.

Natural products represent a cornerstone of pharmaceutical research as they offer a diverse array of chemical scaffolds and bioactive substructures. For example, our laboratory has shown the natural product toosendanin to be a potent inhibitor of BoNT/A and E via disruption of LC translocation into the cell.¹⁴ In addition, other scientists have examined natural product extracts of plants, marine invertebrates, and fungi searching for inhibitors of LC/A, B and E.¹⁵ Remarkably, a few extracts exhibited inhibition of both LC/B and E, although identification of the compound(s) responsible for inhibition remains to be determined.

Chicoric acid, a dicaffeoyltartaric acid, is the main phenolic compound found in the medicinal plant *Echinacea purpurea* indigenous to North America¹⁶. Interestingly, the non-natural isomer L-chicoric acid is one of the most potent, selective, and thoroughly studied inhibitors of the metalloenzyme HIV integrase^{17–18} (Fig 1). Recently, our laboratory has demonstrated that L-chicoric acid, as well as its enantiomer D-chicoric acid, are equally potent inhibitors of the metalloenzyme LC/A.¹⁹

An intriguing aspect of this later finding is that the kinetic data revealed chicoric acid inhibits LC/A unconventionally, i.e. not through direct binding of the catalytic core within the enzyme active site. Instead, inhibition occurs via interaction with the protease distal from the active site. Herein we report that L-chicoric acid is also active against the LC/B enzyme displaying a similar inhibition mechanism.

In an initial screen for inhibition of LC/B by use of our previously described FRET-based system²⁰, chicoric acid was inactive ($IC_{50} > 100 \mu M$). The peptide substrate in this assay was the 40 amino acid peptide **1** (Fig. 2) spanning P22 to P18' (notation of Berger and Schechter²¹). Similarly, chicoric acid was inactive against LC/A up to $100 \mu M$ when activity was monitored with the FRET substrate SNAPtide, a commercially available truncated version of the physiological substrate SNAP-25 (synaptosomal-associated protein 25 kDa) for LC/A. On the other hand, when LC/A activity was monitored by use of a longer 66 amino acid substrate known to make full use of extended enzyme substrate binding interactions, chicoric acid produced partial inhibition that was nonmutually exclusive with an active site-based hydroxamate inhibitor.⁴ Comparable to SNAPtide, our FRET-based assay with peptide **1** is designed to detect inhibitors that compete with the substrate for binding proximal to the active site and could overlook an inhibitor whose mechanism requires an extended substrate more analogous to the natural substrate.

Reexamination for chicoric acid inhibition of LC/B with the extended N-terminal peptide substrate **2** spanning P40 to P18' (Fig. 2) revealed chicoric acid was indeed active. Inhibition was further validated using our HPLC assay²⁰ and chicoric acid displayed a fifty percent of maximum inhibitory activity (IC_{50}) value of $7.5 \pm 1.3 \mu M$ (Fig. 3). Interestingly, we observed partial inhibition by chicoric acid with the LC/B i.e., saturating concentrations of the inhibitor does not produce complete inhibition. The α -constants (fractional velocity at saturating inhibitor) was determined to be 0.5, therefore at saturation, chicoric acid will only produce 50% inhibition (Eq. 1, supplemental material). This partial inhibition by chicoric acid is also displayed with the LC/A.¹⁹

Unusual inhibition behavior such as partial inhibition is suspect and reminiscent of promiscuous inhibition described by Shoichet.^{22–23} Promiscuous inhibition typically is a result of a bulk phase transition with the inhibitor going from monomer to an aggregated state. The presence of a sub-CMC (critical micelle concentration) amount of detergent is very effective at disrupting an inhibitor in the aggregate state. Thus, a method of revealing promiscuous inhibition is to conduct an inhibition profile in the presence of and absence of detergent and observing a shift in the dose response curve. Since our standard HPLC-based assays are incompatible with detergent we examined LC/B activity by use of the continuous FRET-based assay with peptide **2** in the presence and absence of Tween-20 (0.05%) below its CMC. The continuous assay is much less sensitive making analysis difficult but chicoric acid inhibition in the presence and absence of detergent produced similar dose response curves including partial inhibition (Fig. S1, supplemental material).

A series of human VAMP-2 substrates (Fig. 2) were synthesized to investigate the minimum amino acid sequence needed to observe inhibition of LC/B by chicoric acid, thereby suggesting the region where chicoric acid interacts with the enzyme. All peptide substrates contained the identical FRET pair at residues 74 and 77 though each peptide was decreased by several amino acids in relation to peptide **2**, thus producing a series of overlapping peptides (**3–5**). These peptide substrates were monitored for inhibition by chicoric acid in the presence of the LC/B via fluorescence. Interestingly, the only peptide to display inhibition by chicoric acid was the longest peptide **2**, a 58 amino acid stretch of human VAMP-2 (residues 37–94). While this result does not provide definitive proof, it does support the concept that chicoric acid interacts with the LC/B at a location distal from the active site. In the protease literature, binding sites remote from the active site are called exosites.^{24–25} Chicoric acid was inactive against the second largest substrate **3** (residues 43–94), potentially because this substrate does not extend to the binding site of chicoric acid on the enzyme. We can surmise that chicoric acid binds to a region of the BoNT/B protease, which disrupts binding of our largest substrate, specifically in proximity to where the N-terminus region (residues 37–42) of VAMP-2 binds to the LC.

The sequence we have identified to be affected by chicoric acid's interaction with the LC protease is contained in the sequence of human VAMP-2 (residues 33–47) known as the SNARE motif, a common sequence of amino acids found in multiple locations on the SNARE proteins (VAMP-2, SNAP-25 and syntaxin).²⁶ The SNARE motif is found within two positions on VAMP-2 and is composed of amino acids possessing three carboxylates, two hydrophobic, one polar and two random residues; it is predicted to adopt a helical structure.²⁶ Indeed, these motifs are believed to be important for binding to the BoNT LC serotypes. A point in fact is that mutational studies of these motifs in VAMP-2 revealed drastic effects to both the binding and turnover of LC/B.²⁷ In line with our data and previous studies we hypothesize that chicoric acid interacts with LC/B in proximity to where the V1 SNARE motif binds to the LC thereby disrupting binding and catalytic turnover by LC/B.

In conclusion we have discovered chicoric acid to be an inhibitor of LC/B, complimenting chicoric acid's reported ability to inhibit LC/A; hence, a crossover small molecule BoNT protease modulator. We hypothesize chicoric acid is inhibiting the binding of the substrate to the BoNT LC via disruption of SNARE motif binding to the protease. Its IC₅₀ value is modest, but as this natural product's toxicity is low, we believe that chicoric acid could be an excellent scaffold or probe for the production of analogs with improved potencies or delineating exosite interactions. Finally, the SNARE motif that is located within all of SNARE proteins merits additional research as an interesting target for the development of crossover small molecule inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
The chemical structure of L-chicoric acid.

Fig. 2.
 FRET-based synthetic substrates containing the central region of human VAMP-2.

Fig. 3.
Inhibition of LC/B by L-chicoric acid.