Botulinum Neurotoxin A Protease: Discovery of Natural Product Exosite Inhibitors

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Neurotoxins of the anaerobic spore forming bacterium *Clostridium botulinum* are the most lethal human poison.[1] Serotype A (BoNT/A) is the most potent of the several serotypes with an LD_{50} for a 70 kg human of 0.8 μg. Upon cellular internalization of the holotoxin a light chain (LC) 50 kDa zinc metalloprotease is released. Toxicity results from the metalloprotease's site-specific cleavage of the synaptosomal-associated protein preventing acetylcholine containing vesicles from fusing with the presynaptic neuromuscular junction.[2]

Currently, there are no approved pharmacological treatments for BoNT intoxication. Although an effective vaccine is available for immuno-prophylaxis,[3] vaccine approaches cannot reverse the effects after the toxin has reached its target inside the cell. A small molecule pharmacological intervention, especially one that would be effective against the etiological agent responsible for BoNT intoxication, the light chain protease, would be highly desirable and obviate vaccine deficiencies.

The substrate for BoNT/A is SNAP-25 (synaptosomal-associated protein, 25 kDa). The Michaelis complex involves an extensive network of binding interactions ranging from the active site to the opposite surface of the BoNT/A. In the complex, the N-terminal residues of SNAP-25 (147−167) form an α-helix, imbedded in the rear surface of BoNT/A while the C-terminal residues (201−204) form a distorted β-strand, and the spanning residues are mostly extended.[4] Both mutagenesis and kinetics have conclusively shown that the N-terminal α-helix and the C-terminal β-sheet are critical for an efficient substrate binding and cleavage and have been termed α-and β-exosites, respectively.[5] Also, substrate truncation experiments reveal that BoNT/A protease requires a long stretch of SNAP-25 (66-amino acids) to have optimal catalytic activity. Likely, it is the extensive enzyme−substrate binding interactions that make the proteases of BoNTs among the most selective known. This multisite binding strategy incorporating an exceptionally large substrate−enzyme interface area[4] probably accounts for the extreme difficulty in producing potent small molecule inhibitors of the enzyme. In effect, the small molecule must be capable of disrupting these protein−protein interactions.[6] While considerable efforts have gone into identifying active site inhibitors of BoNT/A, no report of a small molecule exosite inhibitor has been communicated.[7] Herein, we provide strong evidence demonstrating that components from the plant Echinacea are potent exosite inhibitors with an unexpected synergistic effect when combined with an active site inhibitor.

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One of the most popular herbs in the U.S. today is the Native American medicinal plant called Echinacea. It has been used for over 400 years to treat infections and wounds and as a general "cure-all". Main components of Echinacea showing biological and pharmacological activity are the phenolic caffeoyl derivatives[8] including **I1**, **I3**, and **I4**, Figure 1. We were intrigued by the structural similarities between the above phenolic caffeoyl derivatives and several known active site inhibitors of BoNT/A (Figure 1), in particular the similarity between **I2**, identified from a high throughput screen,[9] and D-chicoric acid **I1**. Interestingly, the unnatural isomer L-chicoric acid (**I1′**) is a potent inhibitor of the HIV-1 integrase, a metalloenzyme.[10] Consequently we tested these Echinacea components for their inhibition of BoNT/A protease.
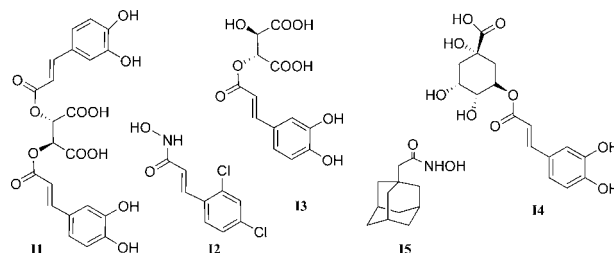

Figure 1. Natural products D-chicoric acid (**I1**), caftaric acid (**I3**), chlorogenic acid (**I4**), synthetic hydroxamates **I2** and **I5**.

Thus, **I1** was evaluated over an extended concentration range with substrate present at K_M (10 μM).[11] Surprisingly, partial inhibition was observed. To evaluate this unexpected kinetic inhibition mechanism, concentrations of **I1** and the substrate (SNAP-25, amino acids 141−206) were varied.[11] A noncompetitive partial inhibition mechanism depicted in Scheme 1 was most consistent with the results. Equation 1 is the rate equation derived from Scheme 1 (Supporting Information) where δ is the fractional V_{MAX} at saturating [**I1**], while K_U and K_C are the uncompetitive and competitive inhibition constants respectively. Figure 2 presents a global fit of **I1** to a matrix of [**I1**] $\times$ [S] from which $\delta = 0.42 \pm 0.04$, $K_U = 1.6 \pm 0.3$ μM, and $K_C = 0.7 \pm 0.1$ μM. A

Scheme 1. Chicoric Acid Mechanism of Inhibition and Eq 1
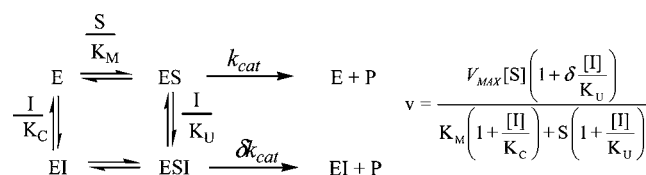
$$v = \frac{V_{MAX}[S]\left(1 + \delta\frac{[I]}{K_U}\right)}{K_M\left(1 + \frac{[I]}{K_C}\right) + S\left(1 + \frac{[I]}{K_U}\right)}$$
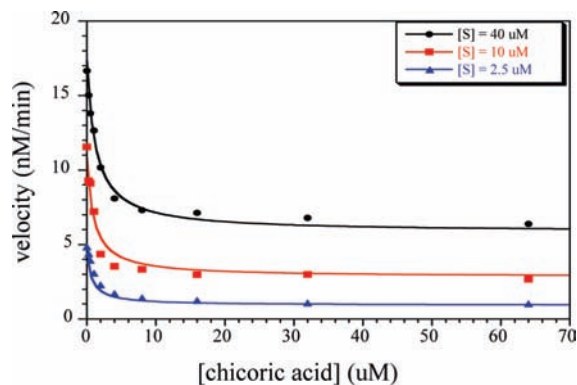
Figure 2. BoNT/A LC catalysis at varied concentrations of substrate and D-chicoric acid. The substrate is an optimized 66 amino acid sequence of the SNAP 25 bracketing the enzyme's active site.

submicromolar competitive inhibition constant makes **I1** one of the tightest binding small molecules yet discovered for BoNT/A. Intriguingly, at saturation, **I1** will only produce 60% inhibition. Consistent with **I1**, the L-chicoric acid **I1'**, **I3**, and **I4** were examined in a similar manner and found to exert the same inhibition mechanism. Interestingly, **I1'** has virtually the same inhibition potency as that of **I1**, although they are enantiomers, while **I3** and **I4** are ~1 order of magnitude less potent (see Supporting Information, Table S1).

Partial inhibition is inconsistent with an inhibitor occupying an enzyme's active site since active site residence of either a substrate or an inhibitor physically precludes occupation by the opposite agent. In other words, if an inhibitor binds within the active site, then, at the saturating inhibitor, the substrate is prevented from binding and catalytic activity falls to zero (producing complete rather than partial inhibition). Therefore, **I1**, **I3**, and **I4** must associate in an exosite some distance from the active site. Such inhibition has been reported for a number of proteases, but not for the BoNTs.[12] Quite likely, the exosite overlaps with a portion of the 66-mer substrate's extended binding region interfering with, but not totally preventing, substrate binding. Simple inhibition experiments do not identify binding site locations. On the other hand, our hypothesized nonoccupation of the enzyme active site by phenolic caffeoyl derivatives may be supported by an inhibitor combination study.[13] Two inhibitors that bind within the active site of an enzyme will, by definition, be mutually exclusive. In a mutually exclusive inhibitor combination, a plot of $1/V_{observed}$ versus the concentration of one inhibitor at varied but fixed concentrations of the second inhibitor will produce a family of parallel lines as a diagnostic pattern. In contrast, if the inhibitors used in combination are mutually nonexclusive, the same plot will produce a family of intersecting lines as a diagnostic pattern. Additionally, the magnitude of the increasing slope with increasing second inhibitor concentration reflects the degree of synergistic binding between the inhibitors. An inhibitor combination study involving chicoric acid is complicated by its partial inhibition; as such, the full rate equation was derived (Supporting Information) and used in the global fit for the combination study.

To confirm our hypothesis of **I1** being an exosite inhibitor, we examined a combination of **I2** and **I5** (Figure 1). Both compounds are optimized hydroxamate inhibitors and have been confirmed by kinetics and crystallographic analysis to bind within the metalloprotease's active site through coordination with the catalytic zinc.[8,10,14] A global fit to the **I2/I5** inhibitor combination experiment was most consistent with the mutually exclusive binding model and clearly visible in the parallel lines of Figure 3A. In contrast,
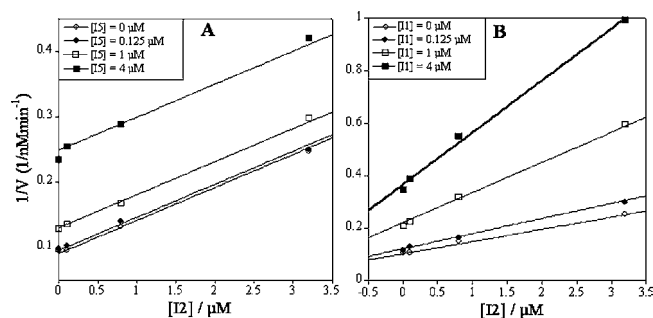

Figure 3. (A) **I2** in combination with **I5** displaying mutually exclusive inhibition. (B) **I2** in combination with **I1** displaying synergistic inhibition.

utilizing the combination of **I2/I1** produced a pattern of intersecting lines demonstrating nonmutually exclusive binding. Interestingly, a global fit of eq 3 (Supporting Information) to the data produced a synergistic or enhancement factor (α) of 1.7 ± 0.3.

We have disclosed a new mechanistic class of BoNT/A zinc metalloprotease inhibitors exemplified by the natural product chicoric acid. A detailed evaluation of chicoric acid's inhibition mechanism reveals that the inhibitor binds to an exosite, displays noncompetitive partial inhibition, and is synergistic with a competitive inhibitor (**I2**) when used in combination. The ability to inhibit an exosite by a small molecule is no simple feat as this requires the disruption of protein−protein interactions.[6] Our work also highlights how natural products could provide a rewarding frontier for the BoNT drug discovery and development. Future research along these lines will be reported in due course.

Supporting Information Available: Full experimental procedures, derivations, and characterization for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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