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Examination of α-exosite inhibitors against *Botulinum* neurotoxin A protease through structure-activity relationship studies of chicoric acid

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ABSTRACT

Botulinum neurotoxins (BoNT) are among the most toxic known substances and currently there are no effective treatments for intraneuronal BoNT intoxication. Chicoric acid (ChA) was previously reported as a BoNT/A inhibitor that binds to the enzyme's α-exosite. Herein, we report the synthesis and structure-activity relationships (SARs) of a series of ChA derivatives, which revealed essential binding interactions between ChA and BoNT/A. Moreover, several ChA-based inhibitors with improved potency against the BoNT/A were discovered.

Botulinum neurotoxins (BoNT), which are produced by the Gram-positive bacterium *Clostridium botulinum*, are among the most lethal known human poisons. The most potent stereotype, BoNT/A, exhibits an intravenous lethal dose of 1–2 ng/kg in humans.[1] Accordingly, BoNT/A is classified as a bioterror threat due to its tremendous toxicity and ease of production. Despite these concerns and the lack of effective countermeasures in the instance of overdose, BoNT/A is also widely used as both a cosmetic and therapeutic.[2]

The lethality of BoNT/A results from intoxication of peripheral neurons, which is mediated through its heavy chain (HC) and light chain (LC).[3] The HC ensures the toxin passes the digestive system, enters circulation, and reaches peripheral neuromuscular junctions, where it is recognized by receptors that mediate endocytosis of the holotoxin.[4] Once translocated into the cytosol, the released LC, a Zn^{2+} dependant endopeptidase, specifically binds and cleaves synaptosomal-associated protein of 25 kDa (SNAP-25). Cleavage of SNAP-25 irreversibly impairs the membrane fusion machinery required for the exocytosis of acetylcholine at neuromuscular junctions. Acetylcholine is essential for neuromuscular transmission; thus, BoNT/A intoxication of nerve endings results in flaccid paralysis and potentially asphyxiation, when paralysis occurs in the respiratory system.[4]

Unfortunately, no effective cure has been developed for BoNT/A intoxication. Available treatments are simply supportive, and patients suffer from long hospital stays requiring mechanical respiration.[5] While an antibody-based antitoxin can be administered immediately following BoNT/A exposure, the antitoxin is not effective once the toxin has been internalized into neuronal cells (<12 h post exposure).[6] Therefore, strategies to antagonize BoNT/A intraneuronally are urgently needed. Small molecule inhibitors offer the sole opportunity for a post-intoxication, intraneuronal therapy.

Earlier, we reported the natural product chicoric acid (ChA) as a non-competitive, partial inhibitor of BoNT/A LC with an IC_{50} = 5.9 μM (Fig. 1A).[7] While the majority of previously reported BoNT/A inhibitors bind the enzyme's active site, ChA binds to the α-exosite, an allosteric region.[8] Our study revealed that the α-exosite plays an integral role in BoNT/A catalytic activity and stability,[9] and is therefore targetable for inhibitor development. In a subsequent study, an *i*-Pr ester analog of ChA (ChA *i*-Pr ester) demonstrated a lower IC_{50} value of 2.7 μM with complete inhibition under saturating conditions (Fig. 1B).[10] Kinetic analysis of ChA and ChA *i*-Pr ester used in combination revealed that the two compounds were mutually exclusive, as parallel curves were observed in the Yonetani-Theorell plot (Fig. 1C).[11] In other words, ChA and ChA *i*-Pr ester were found to bind at the same site of BoNT/A LC. Importantly, this study also demonstrated that synthetic modifications to the ChA scaffold were tolerated by the enzyme.

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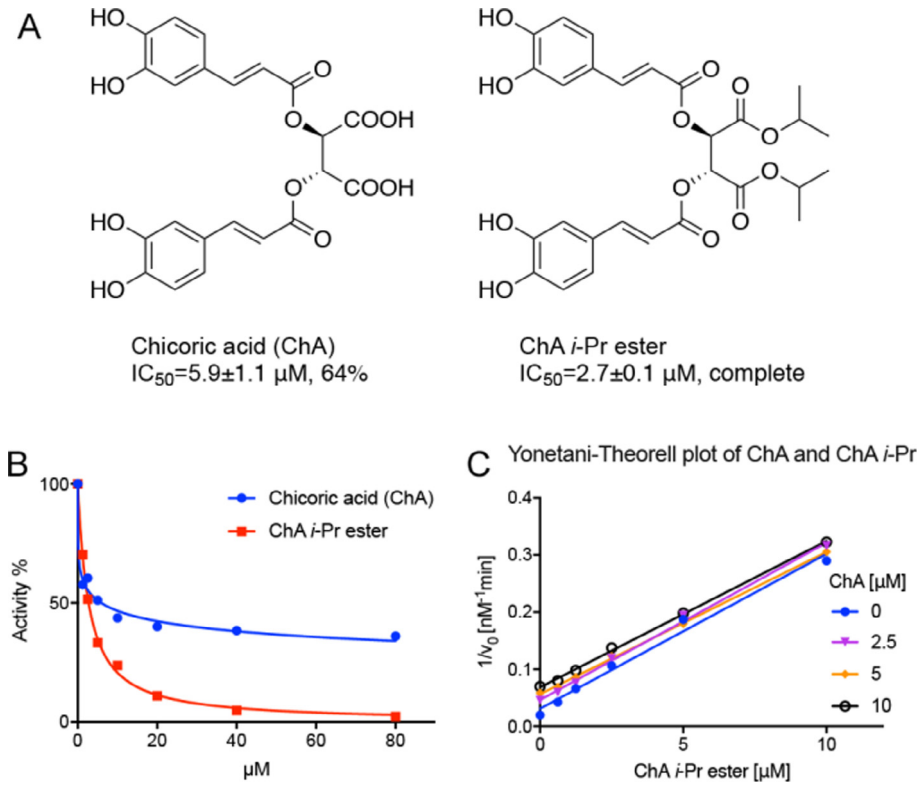
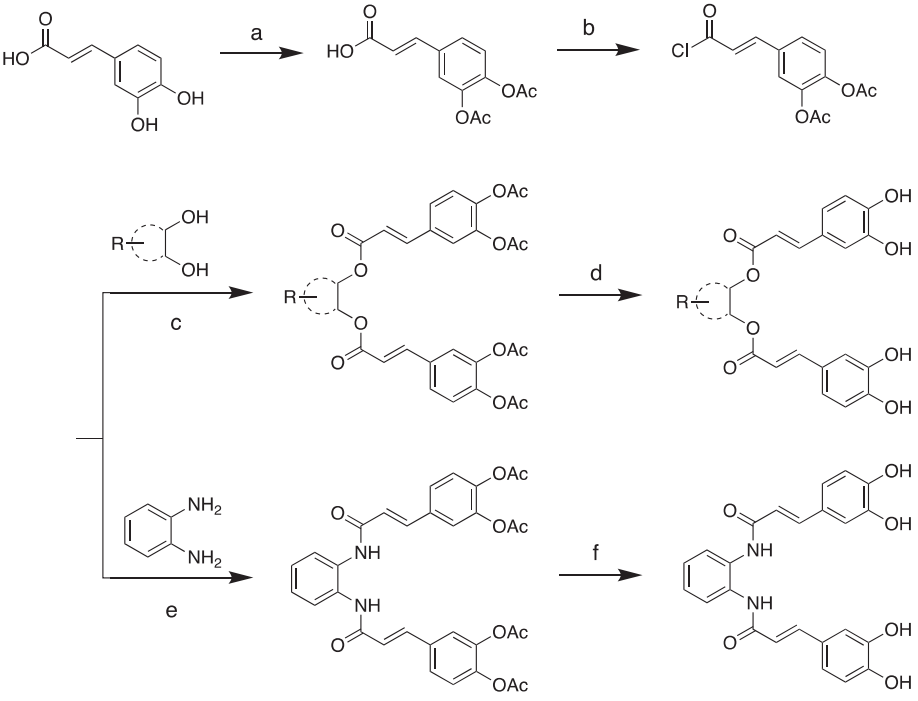


Fig. 1. Structure of Chicoric Acid (ChA) and its *i*-Pr ester analog (ChA *i*-Pr ester) (A). Inhibition curves of ChA and ChA *i*-Pr ester (B). Yonetani-Theorell plot of ChA and ChA *i*-Pr ester.

Though the kinetic parameters and binding site for ChA inhibition have been revealed, a BoNT/A LC – ChA co-crystal structure remains elusive and thus, the specific binding interactions between the enzyme and small molecule remain unknown. To better understand ChA's mechanism of binding, as well as to develop more potent inhibitors, we synthesized a series of ChA derivatives for structure-activity relationship (SAR) studies.

The chemical structure of ChA is defined by two caffeic acid motifs linked by tartaric acid. From our results with ChA *i*-Pr ester, we hypothesized that hydrophobic ester modifications of the



Scheme 1. Synthesis of ChA derivatives with various tartaric ester linkers. Reagents and conditions: (a) Ac_2O, pyr.; (b) $SOCl_2$, benzene; (c) pyr., DMAP, DCM; (d) 2 M HCl, acetone; (e) pyr., DMAP, DCM; (f) 2 M HCl, acetone.