Bioorganic & Medicinal Chemistry Letters 27 (2017) 4956–4959

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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

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ARTICLE INFO

Article history:
Received 28 July 2017
Revised 9 October 2017
Accepted 10 October 2017
Available online 12 October 2017

Keywords:
Botulinum neurotoxins
Chicoric acid
Structure-activity relationship

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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https://doi.org/10.1016/j.bmcl.2017.10.021
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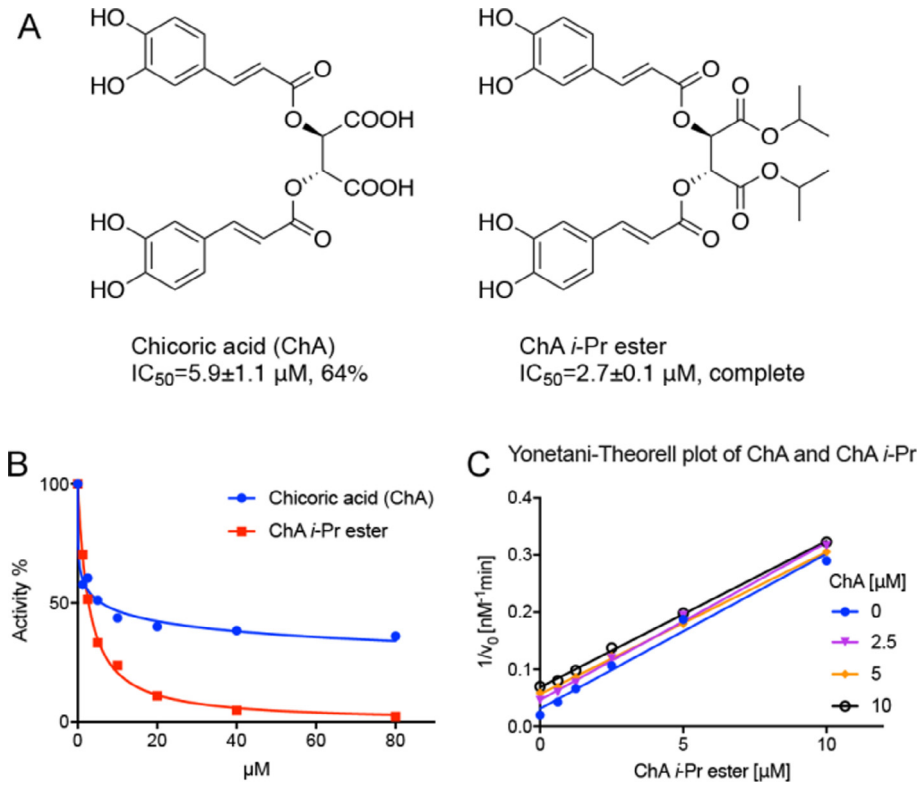
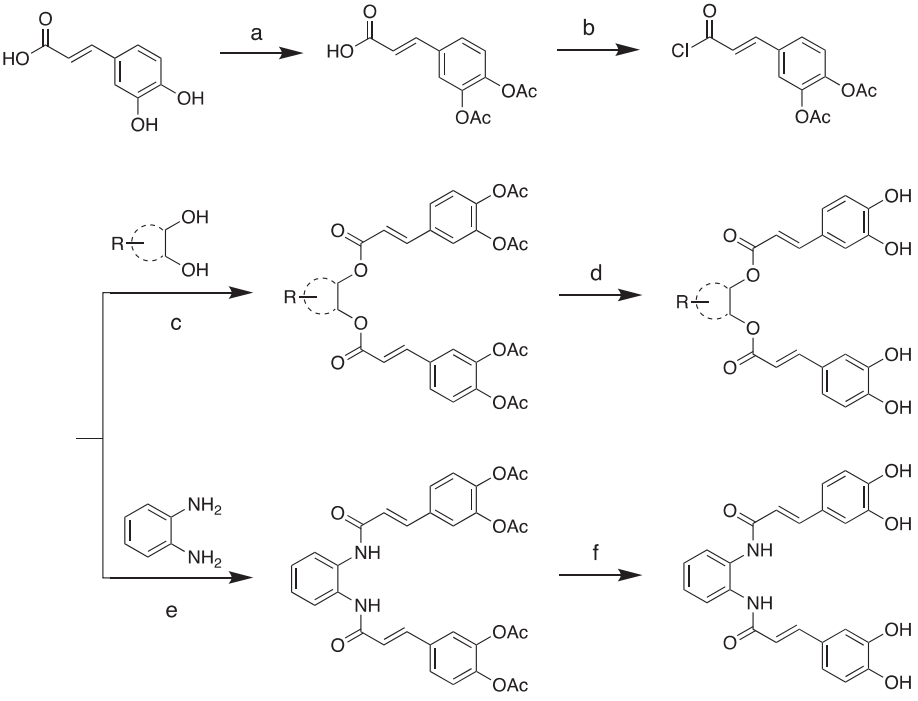


Fig. 1. Structure of Chicoric Acid (ChA) and its *i*-Pr ester analog (ChA *i*-Pr ester) (A). Inhibition curves of ChA and ChA *i*-Pr ester (B). Yonetani-Theorell plot of ChA and ChA *i*-Pr ester.

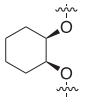
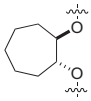
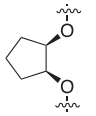
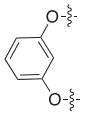
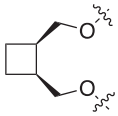
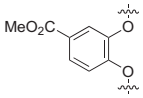
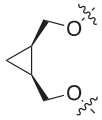
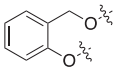
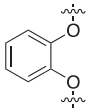
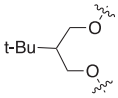
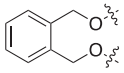
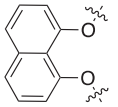
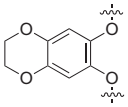
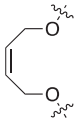
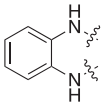
Though the kinetic parameters and binding site for ChA inhibition have been revealed, a BoNT/A LC – ChA co-crystal structure remains elusive and thus, the specific binding interactions between the enzyme and small molecule remain unknown. To better understand ChA's mechanism of binding, as well as to 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.

Table 1
SAR of tartaric acid linker substitutions.[a]

Compound	R	IC_{50} (μM)	Maximum Inhibition (%)	Compound	R	IC_{50} (μM)	Maximum Inhibition (%)
1		3.2 ± 0.6	100	**9**		3.2 ± 0.6	100
2		2.4 ± 0.5	100	**10**		0.56 ± 0.10	100
3		1.7 ± 0.2	100	**11**		0.39 ± 0.05	100
4		0.13 ± 0.03	75	**12**		0.14 ± 0.02	100
5		0.49 ± 0.08	96	**13**		0.13 ± 0.01	100
6		1.1 ± 0.2	100	**14**		0.05 ± 0.01	100
7		0.41 ± 0.21	100	**15**		0.30 ± 0.04	100
8		0.45 ± 0.11	100	**16**		3.3 ± 0.9	100

[a] See the Supporting Information for detailed syntheses.

tartaric acid linker may improve ChA's inhibitory potency. Thus, we first explored a series of ChA derivatives with various linkers, including cycloalkyl-, aryl-, or alkyl-diesters and a diamide (Scheme 1). The synthesized compounds were examined for inhibition of BoNT/A LC activity by LC–MS assay with the 66-mer SNAP-25 substrate, as described in our previous reports.[12] The structures and IC_{50} values are shown in Table 1.

The results revealed that modification of the tartaric acid linker to hydrophobic esters was tolerated. Importantly, all synthesized derivatives maintained inhibition of BoNT/A LC. Not only did most of the analogs have lower IC_{50} values, they also showed complete inhibition of the enzyme under saturating conditions, in comparison to the partial inhibition of BoNT/A LC by the parent, ChA. These findings indicate that hydrophobic linkers containing the dihydroxyphenylacrylate moiety are tantamount for effective BoNT/A LC inhibition. To explain this trend, we hypothesize that a hydrophobic region exists in the α-exosite, which translates to better binding affinities for the more hydrophobic ester-based inhibitors.

Given that the tartaric acid linker was readily modified without compromising BoNT/A LC inhibition, we next hypothesized that the caffeic acid motif, and particularly the four phenol groups, were critical for ChA binding to BoNT/A LC. To investigate the caffeic acid substructure, we prioritized analog **5**, with an IC_{50} = 0.49 ± 0.08 μM, as our lead compound for the following studies. A total of 8 analogs with caffeic acid modifications were synthesized to elucidate the SAR between the caffeic acid motif and BoNT/A LC inhibitory potency (Fig. 2).

We first altered the caffeic acid substructure by truncating the motif to a protocatechuic acid ester, resulting in compounds **17** and **18**. Analog **17** showed moderate inhibition of the BoNT/A LC with an IC_{50} > 20 μM, suggesting that the phenols maintain some LC binding ability, but the truncated protocatechuic acid ester may be too short for the phenols to fully engage the interacting LC residues. Analog **17** was further modified to include a hydrophilic amine group, resulting in **18**. Consistent with our previous results, this compound exhibited no inhibition. We hypothesize that this hydrophilic modification disrupted the aforementioned hydrophobic effect, for which the hydrophobic tartaric esters were optimized. To explore how the caffeic acid phenols interact with the LC, analogs **19**–**24** were synthesized (Fig. 2, see the Supporting Information for detailed syntheses). First, the parent 3,4-dihydroxyphenyl group was reduced to single para- or meta- hydroxy substitution to afford compounds **19** and **20**. Both compounds showed weak inhibition with IC_{50} values = 9.5 and >20 μM, respectively. These results indicate that all four phenols are necessary for BoNT/A LC inhibition, of which the two 4-position phenols are the most important. As a comparison, the four phenols of **5** were acetylated to afford analog **21**, which was inactive against BoNT/A LC as expected. Additionally, compounds **22**–**24**, in which the phenols were replaced with nitro, aldehyde or alcohol substituents, also exhibited no BoNT/A LC inhibition. As many of these derivatives exhibit hydrogen bond accepting properties (OAc, NO_2 and CHO, in particular), we posit that the four phenols are essential hydrogen bond donors in ChA–BoNT/A LC binding interaction.

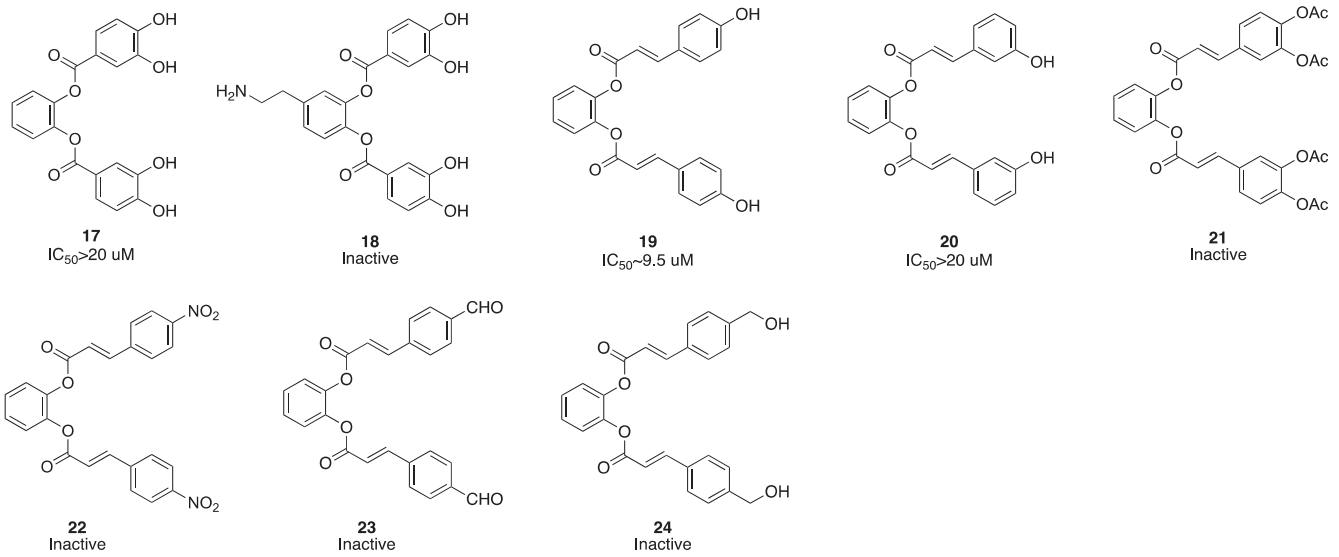


Fig. 2. SAR of caffeic acid modifications.

In sum, by synthesizing a series of ChA derivatives and analyzing their BoNT/A LC inhibitory activity, we revealed that the two caffeic acid motifs are critical for ChA binding to the LC. The interaction between the inhibitors and the LC may occur through hydrogen bond formation involving the phenol groups of ChA as donors. Moreover, substitution of the tartaric acid linker was tolerated and hydrophobic ester modifications were favorable for inhibition. Overall, analogs **1**–**16** demonstrated improved BoNT/A LC inhibitory potency in both IC_{50} value and maximum inhibition efficiency, in comparison to the ChA parent. As a result, analogs **1**–**16** may provide leads for further crystallographic as well as therapeutic studies.

Acknowledgments

We acknowledge the National Institutes of Health (AI117878 and AI119564) for funding. This is manuscript #29551 from The Scripps Research Institute.

A. Supplementary data

Supplementary data (synthesis of the compounds and characterizations) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.10.021.

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