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Benzylidene cyclopentenediones: First irreversible inhibitors against botulinum neurotoxin A's zinc endopeptidase

Kateřina Čapková [a], Mark S. Hixon [b], Sabine Pellett [c], Joseph T. Barbieri [d], Eric A. Johnson [c], Kim D. Janda [a,e,*]

[a] Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[b] Discovery Biology, Takeda San Diego, Inc., 10410 Science Center Drive, San Diego, CA 92121, USA
[c] Food Research Institute, University of Wisconsin, 1925 Willow Drive, Madison, WI 53706, USA
[d] Medical College of Wisconsin, Department of Microbiology and Molecular Genetics, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA
[e] Department of Immunology, The Skaggs Institute for Chemical Biology and Worm Institute of Medical Research (WIRM), The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

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ABSTRACT

A series of benzylidene cyclopentenedione-based inhibitors, acting through covalent modification of the active site of botulinum neurotoxin A light chain metalloprotease, are reported.

Botulinum neurotoxins (BoNTs), the etiological agents responsible for botulism, are the deadliest poisons known.[1] Among the seven serotypes, BoNT/A ranks as the most potent, with a lethal dose for humans of ≈1 ng/kg of body weight. The intoxication event itself may result from improperly stored food, an accidental overdose during a therapeutic treatment or as a result of bioterrorism.[2] For an extensive discussion on the etiology of the BoNTs see Janda and co-workers;[2] however, in brief, the toxin itself consists of a disulfide-linked dimer comprised of a 100 kDa heavy chain and 50 kDa light chain (LC). The heavy chain is responsible for neuronal cell receptor binding and translocation while the light chain is a Zn^{2+}-dependent endopeptidase. The light chain (LC), exclusively cleaves at specific sites of the intracellular soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins; namely, synaptosomal-associated protein (SNAP-25) in the case of BoNT/A LC. This cleavage results in the impairment of neural vesicular docking–fusion machinery and inhibition of acetylcholine release at neuromuscular junctions. At the somatic level, this leads to progressive flaccid paralysis, and, in severe cases, to respiratory failure and death.

Effective therapy against BoNT intoxication is currently lacking. Indeed, the persistence of the activated toxin within a neuron remains the greatest obstacle. Thus, while a pentavalent toxoid vaccine is available, supplies are reserved for high-risk individuals.[2] Alternate countermeasures consist of administration of equine antibodies, yet, this can cause both serious side-effects and has a limited window of application. Additionally, antitoxins are ineffective once BoNT enters the neuronal cells, leaving mechanical ventilation for life support as the only treatment option.[2] In this light, small molecule inhibitors effective after toxin internalization represent a promising strategy, and several efforts have been made to uncover viable lead structures for clinical development.[2] To date, these efforts have been mostly focused on competitive inhibitors targeting the BoNT/A LC. Unfortunately, even if a tight binding inhibitor with the appropriate drug metabolism and pharmacokinetic, (DMPK), properties is achieved, this approach will still suffer from the persistent nature of the toxin. Reversible inhibition of BoNT/A will be transient, solely dependent on the inhibitor's intrinsic DMPK, while BoNT neurotoxin is known to persist within a neuron for weeks to months, albeit by an unknown mechanism.[2]

Although the persistent nature of BoNT/A neurotoxin's protease is a substantial hurdle to overcome, one favorable aspect of BoNT/A intoxication is its nonregenerative nature. In almost all other disease states (infectious agents, cancer, metabolic disorders), the enzyme targeted for therapy is regenerated. In contrast, intoxication with BoNT is a solitary event; BoNT/A LC need to be "killed" once, as there will be no regeneration of the enzyme. This singular intoxication event along with the persistent nature of the enzyme itself makes therapy with an irreversible inhibitor a very attractive

Abbreviations: MMP, matrix metalloprotease.

* Corresponding author. Tel.: +1 858 784 2516; fax: +1 858 784 2595.
E-mail address: kdjanda@scripps.edu (K.D. Janda).

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approach. Mechanistically, an irreversible inhibitor in theory would destroy the cellularly internalized toxin, that is, the light chain protease, as a function of inhibitor exposure (concentration & time) and inhibitor specificity constant (k_{inact}/K_I). Details on the kinetics of irreversible inhibitors are well described in Copeland.[3] Briefly, as depicted in Figure 1, an initial (and reversible) enzyme inhibitor complex forms (EI) that proceeds through a chemical step to an irreversible enzyme inhibitor adduct (EI*). The observable inactivation kinetics by this mechanism is presented in Eq. 1. The method for ranking the potency of irreversible inhibitors is the specificity constant k_{inact}/K_I.

Irreversible inhibitors acting through covalent modification of the active site of the target enzyme have been generally shunned by the pharmaceutical industry mainly due to perceived toxicity resulting from off-target interactions of the drug or its metabolites;[4] however, it is very interesting to note that of the currently marketed drugs within the United States that target enzymes approximately 35% of those enzymes are irreversibly inhibited by the drug.[4]

Continuing our research toward an effective therapy for BoNT intoxication, herein we describe the synthesis and evaluation of a series of BoNT/A LC covalent inhibitors. In our previous work, by use of a hydroxamic acid warhead, we had developed a series of competitive small molecule inhibitors that exploit the relatively shallow P1 binding pocket of BoNT/A LC.[5–7] In our current tactic, we make use of these same binding elements to produce relatively potent affinity labels by replacing the hydroxamate with a cyclopentenedione warhead. Thus, we designed benzylidene cyclopentenedione derivatives 1–3 depicted in Figure 2. In the original hydroxamate series, analogs of both **1** and **2** (**1a** and **2a**, respectively) displayed comparable submicromolar potencies, while the hydroxamate analog of **3** (**3a**) was significantly weaker with an IC_{50} of 41 μM when assayed with BoNT/A LC (Fig. 2).[7] Compounds **1**–**3** were synthesized from the corresponding benzaldehydes in a single step, albeit in moderate yields (Supplementary data).[8]

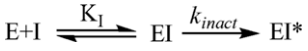


Figure 1. The kinetic mechanism of inhibition by an irreversible inhibitor.

Inhibitors were evaluated (Supplementary data) via a two step assay consisting of an enzyme–inhibitor pre-incubation period followed by dilution with catalytic initiation via substrate addition. Quantitative analysis of BoNT/A LC catalysis was determined as described in Čapková et al.[9] All concentrations of the inhibitors examined produced first order inactivation profiles with respect to BoNT activity. From a secondary plot of the first order rate constant $k_{(observed)}$ versus compound concentration we obtained the specificity parameter k_{inact}/K_I (Eq. 1):

$$\frac{v}{V_0} = \frac{E}{E_0} = e^{-k_{obs}t} \quad \text{where } k_{obs} = k_{inact}\frac{[I]}{[I] + k_I}$$
$$\text{but as } \frac{I}{K_I} \to 0 \quad k_{obs} = \frac{k_{inact}}{K_I}[I] \tag{1}$$

Equation 1: Enzyme activity in the presence of an irreversible inhibitor as a function of time and inhibitor concentration.

The results are summarized in Figure 2. Following the trend of the hydroxamate series,[7] **1** and **2** display comparable affinities for the active site of the enzyme, resulting in similar k_{inact}/K_I values of 520 and 580 $M^{-1}\,s^{-1}$, respectively. Compound **3**, on the other hand, displayed a decreased affinity for the active site of BoNT/A LC as reflected in the significantly lower k_{inact}/K_I value of 80 $M^{-1}\,s^{-1}$ and consistent with its hydroxamate counterpart.

In order to establish the persistence of enzyme inactivation, that is, susceptibility of the EI* complex to hydrolytic rescue, BoNT/A LC was inactivated with **1** (Supplementary data). Following inactivation, the enzyme was exhaustively dialyzed for 16 h, then assayed for recovered activity. Within our limit of quantitation no activity was recovered thus any rescue mechanism must have a half-life of more than 7 h.

We attempted to identify which nucleophilic amino acid within the active site generates the covalent adduct (we suspected Tyr366 given its location within the active site and proximity based on crystal structure[6] to the pentenedione warhead). After pre-exposure of **1** with the BoNT/A light chain sufficient to inactivate the enzyme, the mixture was subjected to tryptic digestion followed by mass spectral analysis via Matrix Assisted Laser Desorption Ionization (MALDI). A series of tryptic digest conditions were examined as well as MALDI conditions, however, no strong fragment

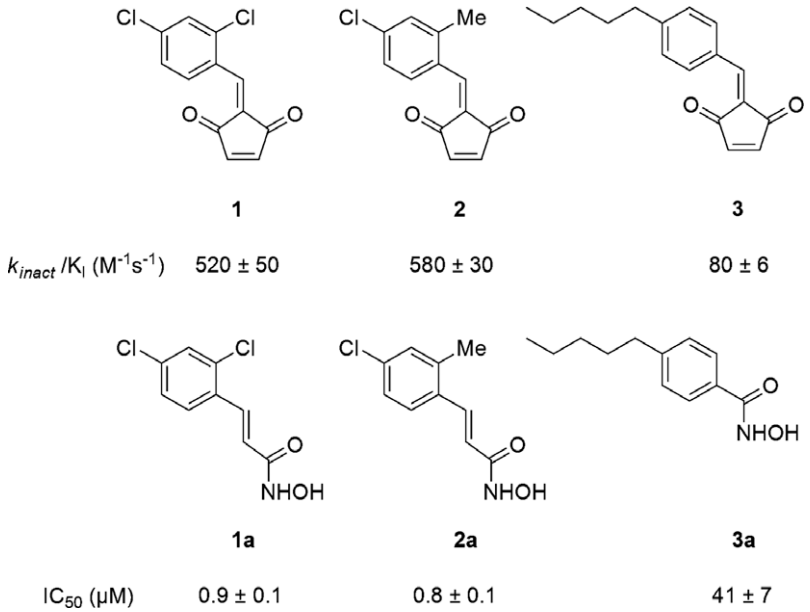
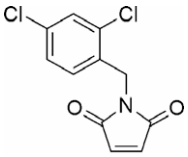


Figure 2. Structure and activity of BoNT/A LC irreversible inhibitors and their hydroxamate counterparts.



4

Figure 3. Structure of maleimide derivative **4**.

signals were uncovered, that is, none of the ionized peptide fragments observed were consistent with cyclopentenedione adduct addition. On the other hand, only 40% of the enzyme peptide sequence was identified by MALDI and Tyr366 was not within this region. Most likely the inhibitor adduct resided in the remaining poorly ionizing 60% of the protein sequence.

In order to evaluate enzyme selectivity, **1** and **2** were screened for off-target inhibition of MMP-2 and MMP-9, both zinc proteases bearing structural similarity to BoNT/A LC.[10] Neither **1** nor **2** showed any inhibitory activity towards MMP-2 (k_{inact}/K_I <33 M^{-1} s^{-1}); however, both derivatives displayed modest inhibition of MMP-9 (k_{inact}/K_I of 61 and 58 M^{-1} s^{-1}, respectively) making it ninefold more selective for BoNT/A LC over MMP-9.

To gain some insight into the specificity of interaction of inhibitors **1**–**3** with BoNT/A LC and to expand our understanding of the enzyme's active site, we synthesized **4** containing the 2,4-dichlorobenzene scaffold, but bearing now a maleimide warhead. (Fig. 3). Compound **4**'s synthesis was straightforward, condensing 2,4-dichlorobenzylamine with maleic anhydride.[11] When assayed with BoNT/A LC, **4** lacked an irreversible step; instead it behaved as a weak competitive inhibitor with an IC_{50} of 80 μM. The inactivity of **4** demonstrates that BoNT/A LC is not generically sensitive to any Michael acceptor even when optimized for active site binding.

Intrigued with the activity of our lead structures against the enzyme, we examined compounds **1** and **4** for their potential to inhibit the biologic activity of BoNT/A1 holotoxin in primary rat spinal cord neurons[12] (Supplementary data). This assay measures target protein (SNAP-25) cleavage inside the cell cytosol as an endpoint. Since it requires all steps of the cellular intoxication process to take place in order to observe SNAP-25 cleavage (i.e., receptor binding, internalization, channel formation, LC translocation into the cytosol, and cleavage of the substrate inside the cytosol), it is sensitive to inhibition at any of these steps. SNAP-25 cleavage was significantly decreased at concentrations of **1** above 600 μM, and was nearly completely inhibited at concentrations above 900 μM (Supplementary data). Unexpectedly, the addition of **4** resulted in a decrease in SNAP-25 cleavage at 62.5 μM, and complete inhibition at 125 μM (Supplementary data). Both **1** and **4** inhibit the biologic activity of BoNT/A1 in neuronal cells and, interestingly, **4** is about 7–10 times more potent than **1** despite its lack of efficacy against a BoNT/A LC enzyme activity assay. The disconnection between enzyme and cellular activity of **4** leads us to posit that the observed cellular activity mechanism could be an indirect effect as SNARE is an abbreviation for soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor. Thus, hexameric ATPase *N*-ethylmaleimide-sensitive factor (NSF) uses energy from ATP hydrolysis to dissociate SNARE complexes after membrane fusion, allowing the individual SNARE proteins to be recycled for subsequent rounds of fusion. Whether **4** intercedes within this complex series of events; now a protease damaged SNARE complex allowing SNARE proteins to be recycled is an attractive hypothesis, which will require further studies including the examination of a series of *N*-ethylmaleimides to sort out this interesting observation.

Finally, as an initial examination of the pharmacological stability of our compounds, we examined the stability of one of our affinity labels in the presence of 1 mM glutathione (approximately physiologic concentration) and in bovine serum. Once again, **1** was chosen as a representative of the series. Unfortunately, subsequent LC–MS analysis showed complete disappearance of **1** within 5 min of incubation with both glutathione and bovine serum. Hence, this would most likely preclude this structure for additional testing in the mouse lethality assay for BoNT/A.

In summation, the properties of BoNT/A intoxication argue for a therapeutic approach engaging irreversible inhibitors. In contrast to the extensive body of knowledge with reversible inhibitors of metalloproteases, and numerous studies with irreversible inhibitors of serine proteases, very little work has appeared on the irreversible inhibition of metalloproteases.[13] We report the first examples of irreversible inhibitors specifically designed against BoNT/A's zinc metalloprotease. Excitingly, these compounds present selectivity against BoNT/A light chain over closely related MMPs, but lack the necessary stability toward serum and glutathione. Interestingly, through these studies we have also uncovered a possible new lead, **4**, providing relatively potent BoNT protection in a cellular assay via a yet to be determined new mechanism of action. Lastly, these studies demonstrate a proof of concept—an important first step for future efforts striving to treat botulinum intoxication.

Acknowledgments

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Supplementary data

Supplementary data (full experimental details: synthesis, inactivation assays, EI* persistence assay, MMP assays, RSC assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.129.

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