Toward the discovery of potent inhibitors of botulinum neurotoxin A: development of a robust LC MS based assay operational from low to subnanomolar enzyme concentrations†

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The development of a sensitive, yet reliable assay for the analysis of botulinum neurotoxin A (BoNT/A) inhibitors is described; using this assay a new protease inhibitor was characterized and found to be one of the most potent inhibitors reported to date.

Among the seven neurotoxins produced by the anaerobic bacterium *Clostridium botulinum*, the serotype A stands out as one of the most lethal poisons known, having a LD_{50} of 0.8 μg for a 70 kg human by inhalation.[1] The toxin itself consists of a 100 kDa heavy chain (HC) linked to a 50 kDa light chain (LC) by one or more disulfide bonds.[2] While the heavy chain is responsible for the translocation and binding, the neurotoxicity results from the catalytic activity of the light chain, a zinc endopeptidase. Specifically, BoNT/A LC cleaves the SNARE (soluble NSF-attachment protein receptor) protein SNAP-25 (a 206 amino acid protein), thus blocking the release of acetylcholine which results in flaccid muscle paralysis and potential death.[3] Although nowadays the historically well known food poisoning cases are extremely rare and BoNT/A is used mostly in cosmetic surgery and medicine for the treatment of muscle spastic disorders,[4] concerns exist regarding the possibility of its misuse as a bioterrorist weapon.[5] Such an attack would be fatal, given that current treatment options for BoNT/A intoxication are quite limited and of a prevalently prophylactic nature.[6] Hence, BoNT/A LC has become a promising target for enzyme inhibitor development,[7] yet there is only a single report of a small molecule non-peptidic inhibitor of this protease with a K_I less than 1 μM.[8]

Closely connected to the search for inhibitors is the development of reliable methods for evaluating their biological activity. In the case of BoNT/A LC, this applies to enzymatic assays for the kinetic analysis of the inhibitors. For high-throughput screening, the method of choice is an assay employing a fluorescence resonance energy transfer (FRET) substrate.[9] The currently most widely used FRET substrate is the SNAPtide™,[10] a substantially truncated and modified sequence of the native BoNT/A LC substrate, SNAP-25. Recently, we reported an optimization for this substrate examining its stability, the impact of detergent and co-solvent effects.[11] While SNAPtide™ proved suitable for high-throughput screening and for crude IC_{50} determinations it is inadequate for finer kinetic analysis such as the evaluation of a kinetic mechanism of inhibition. Consistent with reports on similarly sized substrates,[12] we observed that SNAPtide™ K_M is well above 1 mM, hence any assay with this substrate is conducted under substrate limiting conditions (Fig. 1). Additionally, the low catalytic efficiency of BoNT/A with SNAPtide™ (k_{cat}/K_M approx. 4300 M^{-1} s^{-1}) generally requires enzyme concentrations approaching 100 nM to provide adequate reproducibility.[11] To address these shortcomings, other established HPLC based assays make use of a substrate developed by Schmidt[12] and employ either the BoNT/A LC or the nicked and reduced holotoxin.[14] However, while these assays are functional, they also suffer from drawbacks similar to those of the SNAPtide™ assay due to the nature of the substrate, a 17-amino acid truncated sequence of SNAP-25 with a K_M of 0.8 mM and a k_{cat}/K_M of 650 M^{-1} s^{-1} for the recombinant LC.[14]

We sought to develop an assay wherein the substrate could be varied from below its K_M to at least 5-fold above K_M as well as operating with enzyme concentrations in the low to subnanomolar range. Additionally we desired an assay wherein the run-to-run coefficient variation was less than 10% relative error. We have developed an LC MS based assay employing a truncated but much larger peptide substrate SNAP-25(141-206) (66-mer) thought to contain the majority of the recognition elements of the native substrate.[15] BoNT/A LC cleaves the protein SNAP-25 between the Gln 197 and Arg 198 residues. Using HPLC, the 9 amino acid peptide product of BoNT/A catalysis may be separated from other components within the reaction mixture. Peptide product detection may be accomplished using either UV absorbance or mass spectral analysis. The limit of quantitation (LOQ) for product formation by UV analysis is approximately 100 nM (data not shown). Because of this level of sensitivity, UV analysis requires either high concentrations of enzyme or excessive incubation times; a practical limit of 10 nM enzyme and a reaction time of 1 hour are required. On the other hand, mass spectral detection coupled with the use of a stable isotopically labeled (SIL) internal standard[16] achieved an LOQ for the peptide product of less than 10 nM, allowing a 10-fold reduction in the working enzyme concentration (Fig. 2).

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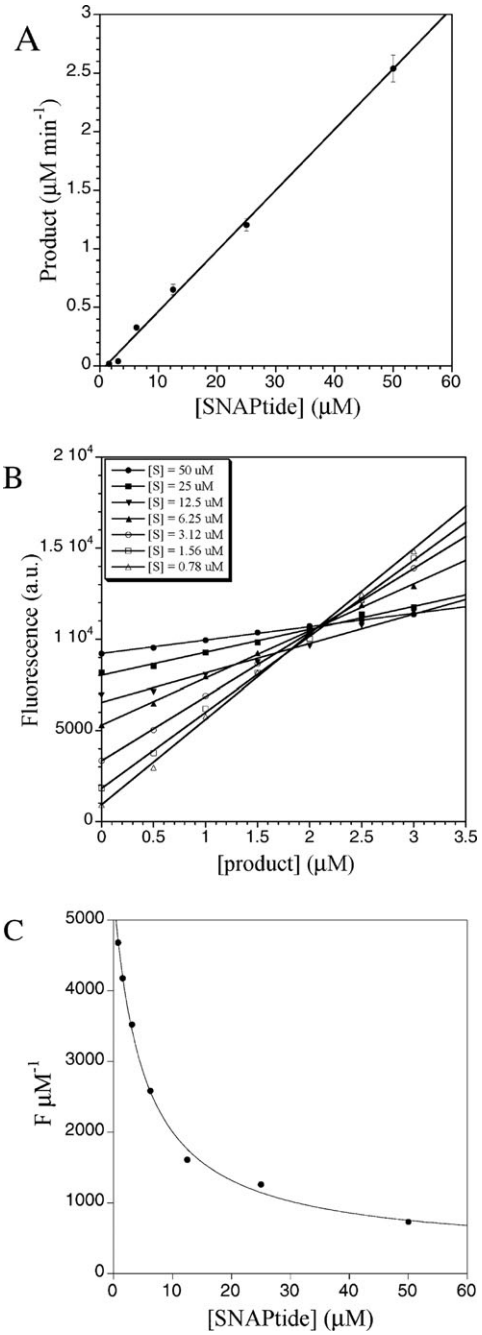

Fig. 1 (A) Determination of BoNT/A catalytic efficiency for SNAPtide™ with inner filter correction.[13] (B) Fluorescence response to increasing product concentration at varied SNAPtide™ concentration. (C) Fluorescence efficiency of hydrolyzed product *vs.* SNAPtide™ concentration.

Adamantane derivatives, most notably the anti-influenza agents amantadine and rimantadine, are widely used in clinical practice for the treatment and prophylaxis of various viral diseases.[17] In this light, we were pleased to identify the 1-adamantane-*N*-hydroxyacetamide **1** (ANH, Fig. 3), from our high-throughput screen using SNAPtide™, as a potential inhibitor. To confirm this "hit" and determine its true kinetic parameters and mechanism of inhibition we describe the development of this LC MS based assay.

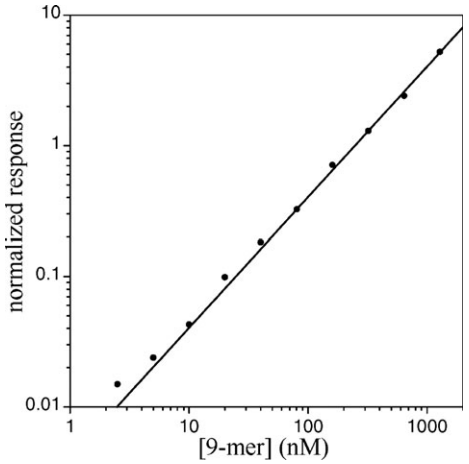

Fig. 2 LC MS dynamic response for 9-mer product.

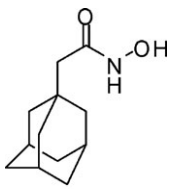

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Fig. 3 1-Adamantane-*N*-hydroxyacetamide (ANH).

We synthesized the reaction product SIL analog, H_2N-Arg-[Ala(1-^{13}C)]-Thr-Lys-Met-[Leu(1-^{13}C)]-[Gly(1-^{13}C)]-Ser-[Gly(1-^{13}C)]-COOH. This was added to aliquots taken from the reaction mixture, and the resulting samples were subjected to LC MS analysis in positive single ion monitoring (SIM) mode. Integration of the peaks corresponding to the product and the SIL internal standard, respectively, afforded product concentration as a function of incubation time. This setting allowed us to work at low enzyme concentrations and relatively small reaction mixture volumes.

The initial rates of product formation were determined for a matrix of substrate and inhibitor concentrations bracketing $K_{M(apparent)}$ and $K_{I(apparent)}$. The resulting velocities were consistent with a competitive kinetic mechanism of inhibition. A nonlinear least squares global fit to the velocities produced a K_I of 460 ± 80 nM. The fit also produced a k_{cat} of 0.33 ± 0.02 s^{-1} and a K_M of 10 ± 2 μM (k_{cat}/K_M approx. 33 000 M^{-1} s^{-1}) for the SNAP-25(141-206) peptide substrate in good agreement with previously determined values (Fig. 4 and 5).[11]

Although the results suggested a competitive mechanism of inhibition, we were concerned about the possibility of aggregation of the inhibitor. This effect has recently been thoroughly investigated by Shoichet[18] and detergents were found to disrupt these nonspecific interactions. However, the use of detergents is very problematic in the LC MS-based assay, so for this particular experiment we turned to the FRET-based assay with SNAPtide as substrate. Gratifyingly, there was no difference in inhibitory activity observed either in the presence or in the absence of detergent, indicating that no aggregation takes place.

To conclude, we have developed an accurate, sensitive and reliable assay for the kinetic analysis of BoNT/A small

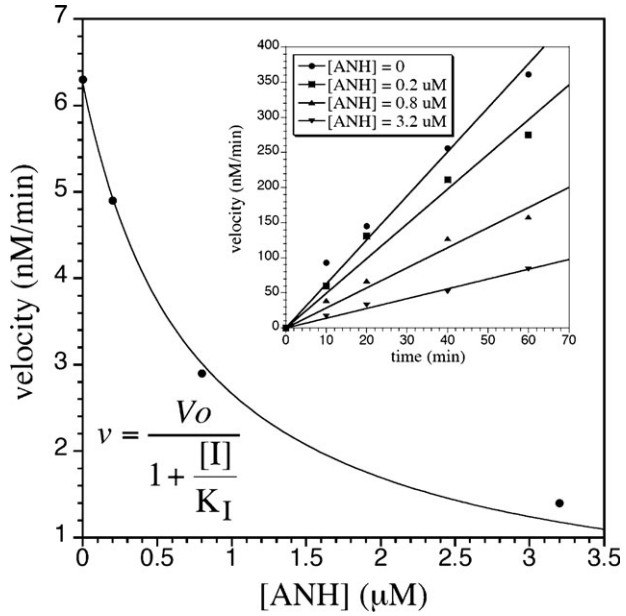

Fig. 4 Dose response curve of ANH at 2.5 μM 66-mer. Inlay: Product formation at timed intervals at 2.5 μM 66-mer and varied concentrations of ANH.

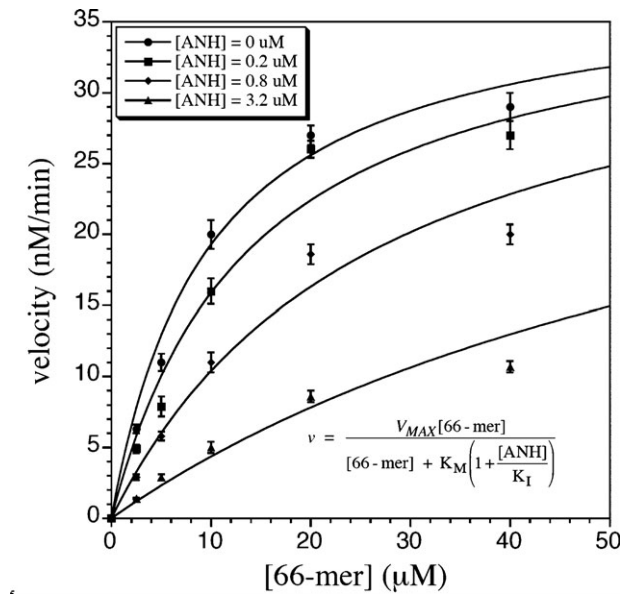

Fig. 5 Global fit of a competitive inhibition mechanism to a matrix of substrate (66-mer) × inhibitor (ANH).

molecule inhibitors. Using this assay we have confirmed that 1-adamantane-*N*-hydroxyacetamide, (**1**, ANH), ranks as one of the most potent small molecule inhibitors of the BoNT/A LC and thus provides a new scaffold for the development of BoNT/A protease inhibitors.

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