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Covalent modifiers of Botulinum neurotoxin counteract toxin persistence

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Abstract

Botulinum neurotoxins (BoNTs) are the most potent toxin known to man and a significant threat as a weapon of bioterrorism. BoNTs contain a metalloprotease domain that blocks neurotransmitter release in nerve terminals, resulting in a descending, flaccid paralysis with a 5–10% mortality rate. Existing treatment options cannot access or neutralize toxin following its endocytosis, so there is a clear need to develop novel therapies. Numerous substrate-based and zinc-chelating small-molecule inhibitors have been reported, however none have progressed to the clinic. This is likely due to the difficulty of reversible inhibitors to achieve sustained inhibition of the toxin, which has a months-long half-life *in vivo*. An alternative strategy to mitigate BoNT persistence is through covalent, irreversible inhibition of toxin function. However, few examples of covalent BoNT inhibitors have been reported. Here, we describe a competition-based screen to identify covalent modifiers of the conserved active-site adjacent cysteine C165 in the BoNT/A serotype. We found that compounds containing cysteine-reactive electrophiles designed to target cysteine proteases failed to bind C165 while selenide compounds were efficient covalent binders of this cysteine. Importantly, covalent modification at C165 resulted in sustained, irreversible inhibition of BoNT/A protease activity. Covalent selenide inhibitors were non-toxic and protective in a neuronal assay of intoxication making them promising new scaffolds for the study of the BoNT/A toxin as well as for the design of novel therapy agents.

Graphical abstract

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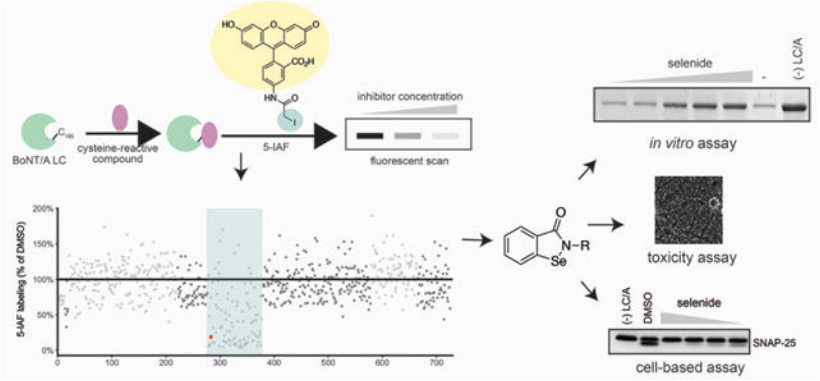
Supporting Information. Supplemental figures, detailed synthetic procedures and compound characterizations can be found in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.



INTRODUCTION

Botulinum neurotoxins (BoNTs) are a family of bacterial toxins produced by the spore-forming, obligate anaerobe *Clostridium* species. BoNTs are the most deadly toxins known to man, with a lethal dose of approximately 1 ng/kg.[1, 2] BoNTs target presynaptic cholinergic ganglia and motor neurons, blocking neurotransmitter release to cause the symmetric, descending, flaccid paralysis characteristic of botulism.[1, 3] The incidence of botulism is rare in human populations, with fewer than 200 cases reported annually in the United States.[1] However, BoNTs are a significant threat as a potential weapon of bioterrorism and are listed as a Category A and Tier 1 Bioterrorism Agent in the United States.[4, 5]

There are seven major serotypes of BoNTs (A–G), of which BoNT/A, B, E, and F are associated with botulism in humans.[6] While the serotypes differ significantly in their primary sequences, all BoNTs are synthesized as a ~150 kDa protein that is proteolytically cleaved into a heavy chain (HC) and light chain (LC). The HC and LC remain linked by a conserved disulfide bond.[6, 7] Upon HC-mediated receptor binding on presynaptic terminals, the toxin enters neurons via receptor-mediated endocytosis. Endosomal acidification induces conformation changes to the N-terminus of the HC, which mediates translocation of the LC into the cytosol. All BoNT LCs are zinc-dependent metalloproteases bearing a conserved HExxH catalytic zinc-binding motif. These proteases cleave members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family of proteins, including synaptosome associated protein 25 (SNAP-25; BoNT/A, E, and C), syntaxin (BoNT/C), and VAMP1/2/3 (BoNT/B, D, F, and G).[6, 7] SNARE proteins act as molecular zippers to facilitate membrane fusion of synaptic vesicles containing neurotransmitter with the neuronal membrane.

Therapies to treat botulism are limited. Antibiotics are not used except in cases of wound botulism, as vegetative bacteria are rarely ingested in cases of adult gastrointestinal botulism, and antibiotics are not efficacious in infant botulism.[1] Two antitoxins exist to provide passive immunity to infected patients, the equine-derived heptavalent botulism antitoxin for adults and the human-derived botulinum immune globulin, called BIG-IV or BabyBIG, for infants.[1, 8] While these antibodies effectively neutralize toxin in circulation, they cannot access BoNTs after they have been internalized into neurons, so their efficacy decreases as intoxication progresses. Therefore, therapies are largely supportive and depend

on the severity of symptoms, with death occurring in 5–10% of patients, usually via suffocation due to paralysis of upper airway muscles and the diaphragm.[1, 3]

To address the need for improved botulism therapies, considerable effort has been focused on small-molecule therapies, but so far none have progressed to the clinic.[9] Of the many reports of small-molecule BoNT inhibitors, most are substrate mimics or zinc chelators.[9] One early and potent example of a zinc-chelating compound is 2,4-dichlorocinnamic acid hydroxamate, with an IC_{50} of 410 nM against BoNT/A LC in an *in vitro* assay.[10, 11] Interestingly, while this compound was highly toxic to cells and displayed no protective effect in cell-based assays below toxic levels, it showed slight efficacy in a mouse toxicity bioassay.[12]

The lack of success of small-molecule inhibitors may be explained by the difficulties of addressing two key challenges in the design of BoNT inhibitors. First, therapeutics must be cell-permeant to be effective later in the course of intoxication when BoNTs are intracellular. Second, BoNTs are known to be highly persistent in neurons—BoNT/A can persist 4–6 months in human neurons[13, 14]—so effective therapies must achieve sustained inactivation of the toxin. This persistence highlights the limitation of reversible inhibitors that are potent *in vitro* but that cannot achieve sustained inhibition in clinically relevant models.

An alternative method to mitigate BoNT/A persistence in neurons is through the development of irreversible inhibitors. Irreversible inhibition is an attractive strategy because a small-molecule inhibitor that covalently targets the LC would permanently inactivate the toxin. Further, botulism typically arises from a one-time exposure to toxin, so permanent inactivation of the total pool of toxin would be expected to prevent paralysis. The few examples of irreversible BoNT inhibitors include benzimidazole acrylonitriles and benzoquinones, both of which are hypothesized to bind to the active site-adjacent reactive cysteine, C165.[15, 16] However, the site of modification was not verified via mass-spectrometry or co-crystallization. A functional study of C165 found that the residue is not required for proteolytic acitivity, though the C165A mutant of BoNT/A shows delayed toxicity and decreased potency.[17] Further, C165 is hypothesized to have a low pK_a and thus may be nucleophilic enough to react with cysteine-reactive warheads.[15, 17] A co-crystal of BoNT/A LC with the cysteine-reactive compound 2-aminoethyl methanethiosulfonate (MTSEA) served as a proof-of-concept for covalent modification of C165.[17] However, the high reactivity of the sulfonyl electrophile makes MTSEA unsuitable for cell-based and *in vivo* studies. Regardless, these studies highlight that C165, due to its reactivity and proximity to the active site, is an attractive target for the development of irreversible inhibitors.

Here, we describe the development of a competition assay with a cysteine-reactive activity-based probe to identify covalent modifiers of C165. We identify a novel class of small-molecule selenide compounds as covalent modifiers of the active site-adjacent C165 residue in BoNT/A LC that act as permanent inhibitors of protease activity. This irreversible inhibition mechanism enabled protection of primary neurons against toxin that was not achieved by a prior published reversible inhibitor of BoNT activity.

RESULTS

The cysteine-reactive compound 5-iodoacetamide fluorescein competitively labels C165.

BoNT/A LC contains two free cysteine residues within the protease domain, C134 and C165, with the third residue near the C-terminus, C430, engaging in a disulfide bond with the HC (Figure 1A). Analysis of the LC crystal structure (PBD 4EJ5)[17] shows that C165 lies close to the catalytic zinc, with an approximate distance of 8.5 Å (Figure 1B). To identify compounds that covalently target C165, we developed a screen based on competition with the generic thiol-reactive compound 5-iodoacetamide fluorescein (5-IAF; Figure 1C). Treating wild-type BoNT/A LC with 5-IAF and resolving the labeled protein by SDS-PAGE gave a robust fluorescent signal (Figure 1D). To determine the BoNT/A LC residue modified by 5-IAF, we generated three mutant forms of BoNT/A LC: C134A, C165A, or C134A/C165A. When treated with 5-IAF, the resulting fluorescent signal from each of the single mutant constructs was approximately 50% of the wild-type signal, while the double cysteine mutant did not label with 5-IAF (Figures 1D and 1E). Thus 5-IAF binds to both free cysteine residues in the LC, and labeling is dependent on the presence of the cysteine residues.

To evaluate 5-IAF labeling as a readout for cysteine modification, we designed a competition assay, first testing 5-IAF labeling with the generic cysteine-reactive compound *N*-ethylmaleimide (NEM; Figure 1C). Pre-treatment of wild-type or C134A BoNT/A LC with NEM before labeling with 5-IAF resulted in a dose-dependent reduction in 5-IAF fluorescent signal (Figures 1D and 1E), thus validating the competition assay for screening specific binders of C165A.

Selenides are the preferred electrophile for modification of BoNT/A LC C165.

To screen for compounds that modify C165, we utilized the C134A construct of BoNT/A LC. Pre-treatment and labeling conditions were optimized in the gel-based competition assay to achieve a high signal-to-noise ratio between modified and unmodified C165 (Figure S1). We screened a library of approximately 700 compounds bearing a variety of cysteine-reactive electrophiles including chloroacetamides, acrylamides, aldehydes, epoxyketones, vinyl sulfones, sulfones, vinyl ketones, acyloxymethyl ketones, and selenides, for competitive modification of C165 (Figure 2A). For this screen, BoNT/A LC C134A was pre-treated with 10 μM of each compound for 30 minutes, labeled with 5-IAF, resolved by SDS-PAGE, and analyzed for fluorescence intensity (Figure 2A). Strikingly, modification of C165 was consistently achieved only by compounds bearing a reactive selenium. Nearly half (43%) of the selenides tested reduced 5-IAF labeling below 25%. Neither longer pre-incubation times nor higher compound concentrations further decreased labeling for other compound classes (Figure S2). We hypothesize that this specificity may be due to the low reactivity of C165. While Li and colleagues hypothesized that C165 exists as a thiolate anion and has a low pK_a due to its proximity to R231,[15] it is not as nucleophilic as active-site cysteines and may therefore require highly reactive electrophiles for efficient modification.

The identified selenides included the parent compound ebselen (2-phenyl-1,2-benzoselenazol-3-one; Figure 2B), which has been found to be safe in human clinical trials

for a number of diverse indications. Ebselen has been reported to protect against BoNT intoxication *in vivo*, although it was hypothesized to act through the compound's antioxidant properties rather than as a result of its direct action on the toxin.[18, 19] Ebselen has also been previously reported as a cysteine-reactive compound capable of covalently modifying the active-site cysteine of the *Clostridium difficile* toxins TcdA and TcdB,[20] therefore we hypothesized that it could also act to block BoNT enzyme activity directly by covalent modification of C165.

To confirm that ebselen covalently modifies C165, we performed mass spectrometry experiments and found that pre-incubating BoNT/A LC C134A with ebselen versus vehicle control resulted in an increase of 270 in the deconvoluted mass spectrum, corresponding to ebselen modification (molecular weight 274.98) at C165 (Figure 2B). To further confirm that modification occurred solely at cysteine residues within the protease domain, we repeated the mass spectrometry experiment with BoNT/A LC wild-type and the cysteine-free mutant, C134A/C165A. When BoNT/A LC wild-type was incubated with ebselen, we observed single and double modifications, corresponding to ebselen modification at both cysteine residues (Figure 2C, Figure S3). In contrast, the BoNT/A LC C134A/C165A double mutant showed no modification upon ebselen incubation (Figure 2C, Figure S3). These data confirm that ebselen exclusively labels the two cysteine residues of BoNT/A LC.

Covalent modifiers of C165 inhibit BoNT/A LC protease activity *in vitro* in a cysteine-dependent manner.

Based on the proximity of C165 residue to the protease active site (Figure 1B), we hypothesized that modifiers of C165 could inhibit protease activity. To measure *in vitro* activity of BoNT/A LC, we developed a fusion protein containing 66 amino acids of the physiologic substrate SNAP-25 flanked by the fluorescent proteins Clover and mRuby2 (CS25R, Figure 3A). Cleavage by BoNT/A occurs between glutamine 197 and arginine 198 residues in the native substrate, which can be detected in gel-based assays or optically via a loss of Förster resonance energy transfer (FRET) signal between the two fluorescent proteins. We first treated CS25R with wild-type and mutant variants of BoNT/A LC and measured residual full-length CS25R substrate by SDS-PAGE (Figure 3B). Time-dependent cleavage was observed for the wild-type protease, while the C165A mutant displayed delayed kinetics consistent with previous reports.[17] Mutation of one of the three residues coordinating the active-site zinc ion, E262Q, resulted in complete loss of protease activity, while the C134A construct used in the 5-IAF competition screen displayed cleavage kinetics similar to the wild-type enzyme (Figure 3B).

We first tested the ability of the parent selenide, ebselen, to inhibit protease activity. Pre-treatment of BoNT/A LC with increasing concentrations of ebselen for 30 minutes before addition of CS25R resulted in dose-dependent protection of the full-length CS25R from proteolytic cleavage as measured by gel-based analysis (Figures 3C and D). FRET experiments were carried out over a range of ebselen pre-treatment times, where co-addition of ebselen and the substrate led to an IC_{50} value of 410 nM (Figures 3E and F). Potency was increased with increasing pre-treatment time, with nearly complete inhibition observed at concentrations as low as 200 nM following 30 minutes pre-treatment (Figure 3F), closely

matching the activity achieved in the gel-based activity assay (Figure 3D). These results validate the use of CS25R FRET as a screen for BoNT inhibition.

To determine whether covalent modifiers of C165 also inhibit BoNT/A activity, we screened the entire sub-library of 106 selenide compounds in the FRET-based activity assay at 100 nM (Figure 4A) and 1 μM (Figure S4A). Activity was quantified by normalizing the rate of substrate cleavage in the presence of inhibitor by the rates observed for DMSO controls (100% activity) and the rates observed for toxin-free controls (0% activity). We classified hit compounds as those that reduced activity below 50% as compared to DMSO controls and identified 9 and 35 such hits at 100 nM and 1 μM doses, respectively. All compounds identified as inhibitors at 100 nM also showed inhibition at 1 μM (Figure S4B). We compared the results of the FRET inhibition and the 5-IAF competition screens to determine the association between covalent modification and enzyme inhibition. Nearly all of the hit compounds identified from the FRET screen similarly modified C165; 100% and 97% of the compounds identified as FRET hits at 100 nM and 1 μM, respectively, also reduced C165 modification by 5-IAF by 50% or more in the competition screen (Figure 4B). Further, while the parent compound ebselen was identified as a potent inhibitor at 1 μM (Figure 4B, right), it was substantially less potent than other analogs in the screen (Figure 4B, left). Finally, we tested whether inhibition of protease activity was dependent on the presence of the two cysteine residues in the BoNT/A LC protease. While ebselen displayed potent and dose-dependent inhibitory activity with the wild-type protease, it did not inhibit activity of the C134A/C156A double cysteine mutant at any dose (Figure 4C). Thus, BoNT/A LC inhibition is cysteine-dependent and correlates with C165 modification.

Ebselen achieves persistent inactivation of BoNT/A LC.

One challenge to the development of small-molecule BoNT/A inhibitors is the long half-life of the protease within host neurons. We hypothesized that the selenide covalent modifiers would mitigate this challenge, as covalent modification would allow for permanent inactivation of the protease. To test this, we compared the parent selenide ebselen to a prototypical potent, but reversible, zinc-chelating inhibitor 2,4-dichlorocinnamic acid hydroxamate (Figure 5A), with a reported IC_{50} of 410 nM in a FRET-based activity assay.[11] Both compounds inhibited cleavage of CS25R by BoNT/A LC in gel-based assays in a dose-dependent manner (Figures 5A and B). Importantly, ebselen pre-treatment resulted in sustained inhibition of BoNT/A LC even after removal of unbound or reversibly bound molecules by dialysis, whereas BoNT/A LC pre-treated with the hydroxamate regained full activity after dialysis (Figure 5C). We next quantified the irreversible inhibitor kinetic parameters for ebeslen and calculated an apparent K_I of 110 nM with a k_{inact}/K_I of 23,000 M^{-1} sec^{-1} (Figure 5D), making ebselen approximately 100-to 1000-fold more potent than the two other confirmed covalent modifiers of C165.[17] Together, these data highlight the ability of ebselen to achieve potent, permanent inhibition of the BoNT/A LC protease activity.

Covalent modifiers inhibit full-length BoNT/A in primary neurons.

Given the potency and long-lasting effects of the selenide compounds *in vitro*, we next sought to test their efficacy in primary neurons. We selected compounds that were both strong covalent modifiers of C165 and potent inhibitors *in vitro*. The compounds were first

tested in healthy primary neurons over three days to eliminate any compounds that showed signs of general toxicity to the target cell type. Compounds that displayed significant toxicity below 100 μM, based on morphological changes to the neurons, were excluded from further analysis (Figure 6A and Figure S5).

We first evaluated the ability of ebselen to inhibit BoNT/A LC-mediated cleavage of native SNAP-25 protein in brain detergent extract. BoNT/A LC was pre-treated with ebselen before incubation with brain detergent extract. Reactions were then subjected to immunoblot analysis using an antibody that recognizes both intact and cleaved SNAP-25. Ebselen showed dose-dependent protection of native SNAP-25 cleavage and strong efficacy at low micromolar concentrations (Figure 6B). We next tested the efficacy of the non-toxic selenide compounds at a single concentration (Figure S6). Compounds displaying efficacy at 5 μM were included for further study. Using this criterion, four compounds (MG35, MG9, MG41, MG2) were chosen for further analysis along with the parent compound ebselen (Figure 6A, Table 1). As controls, we also included the reversible hydroxamate inhibitor and a non-toxic selenide compound, MG22, that failed to covalently modify C165 or inhibit BoNT/A LC activity. In neuronal lysate, these compounds showed a dose-dependent protection of SNAP-25 cleavage and strong efficacy, with MG2 displaying nearly complete protection at 1 μM (Figure 6C). As expected, MG22 failed to protect SNAP-25 cleavage as did the reversibly binding hydroxamate, indicating the importance of irreversible C165 modification for protective effects. Together, these data show that the selenide hits inhibit cleavage of native, full-length SNAP-25 by BoNT/A LC in a complex milieu containing numerous proteins.

Finally, we tested whether selenide compounds could be used to inhibit full-length BoNT/A in cultured primary neurons. We pre-treated full-length BoNT/A with each compound before adding the mixture to culture medium cultures for 14 hours. Neurons were then harvested, with intact and cleaved SNAP-25 visualized via western blot. Ebselen and each of the four top hit compounds completely protected against SNAP-25 cleavage at concentrations as low as 17 μM, whereas the hydroxamate and MG22 compound showed no efficacy (Figure 6D). Thus, the selenide hits permanently disable full-length BoNT/A in primary neuronal culture.

DISCUSSION

There is considerable interest in developing small-molecule inhibitors for BoNT LCs due to current limitations in botulism treatment strategies as well as the potential for BoNTs to be used as weapons of bioterrorism. Current treatment strategies rely on anti-toxin antibodies, however these agents are rendered useless once the toxins enter cells. Therefore, a small molecule anti-toxin agent has the potential to dramatically improve therapy. Multiple potential classes of small molecule inhibitors have been identified and reported in the literature. Most compounds are based on zinc chelators or mimics of the native peptide substrates. Despite these efforts, few reported small-molecule inhibitors of BoNT/A LC have been tested *in vivo* and none have advanced to human clinical trials.[9] This is likely due to the fact that, while many of the reported compounds can potently block BoNT protease activity *in vitro*, their reversible binding mode prevents the long-term inhibition of enzyme activity required to counteract the long half-life of toxins within neurons. Here, we utilized a

competition screening method focusing on covalent modification of C165 to identify a set of small-molecule selenides that irreversibly inhibit BoNT/A protease activity. Interestingly, one of these hits is the well-studied compound ebselen that has been extensively tested in human clinical trials for a number of diverse indications. Furthermore, we identify non-toxic analogs of ebselen with enhanced potency against BoNT/A that are protective in primary neurons. These compounds are promising new leads for further development in the treatment of botulism.

An intriguing observation from our screening results was the strict preference of C165 for selenide compounds over more commonly used cysteine-reactive electrophiles such as acrylamides, epoxyketones, and vinyl sulfones.[21] We suggest a few possible explanations for this preference. First, detailed structural analyses of the C165 mutant show that this residue has reduced accessibility, though there is some local flexibility to accommodate binding,[17] raising the possibility that the larger, peptidic cysteine-reactive compounds that typically bind in more solvent-accessible protease active sites were too big to be accommodated in this pocket. Co-crystallization efforts with BoNT/A LC and selenides to confirm this hypothesis or offer an alternative explanation are active areas for future work. Second, though previous work has shown C165 to be important for catalytic efficiency of BoNT/A, potentially through an interaction with R231 during catalysis,[17] it is likely to be less nucleophilic than active site cysteines traditionally targeted with these cysteine-reactive electrophiles. Indeed, the only other compound confirmed through mass spectrometry or co-crystallization to modify C165 are MTSEA and 3-aminopropyl methanethiosulfonate (MTSPA), compounds that contain the highly reactive sulfonyl electrophile.[17] Interestingly, our data show that the overall potency of the selenium containing molecules does not track with their overall electrophilicity suggesting that some element of molecular recognition within the region surrounding C165 is important for covalent modification and target inhibition (Table S1). Although one limitation to highly reactive electrophiles is their lack of specificity and therefore potential for host toxicity as a therapeutic agent, selenide compounds were generally non-toxic to host cells and the parent molecule ebselen has been tested in multiple human clinical trials (clinicaltrials.gov identifiers NCT01452607, NCT02819856, NCT01444846, NCT01451853, NCT02779192, and NCT02603081) and proven safe for humans.[22] In fact, due to the overall low toxicity of the molecule, clinical studies have been performed using as much as 1.2 gram daily doses of the drug for 21 days (Clinical trial NCT0260308). These high doses of the drug result in systemic exposure of millimolar levels of the drug, which suggests that even with overall weak potency, the drug could provide efficacy without issues of toxicity.

Selenides were initially included in this focused screen of cysteine-reactive compounds due to our recent work showing that the parent compound ebselen covalently modifies the active site cysteine of toxins A and B produced by the bacterium *Clostridium difficile*.[20] Interestingly, ebselen had been previously reported as protective against BoNT activity, but was hypothesized to function through a host-centric mechanism of thioredoxin and thioredoxin reductase inhibition that prevented the reduction of the disulfide bond between the LC and the HC.[18] Here we identify an alternative or additional explanation for the benefit of selenides in the treatment of botulism.

The fact that ebselen covalently modifies BoNT/A toxin in addition to two other *Clostridial* exotoxins suggests that broad-spectrum anti-virulence compounds may serve as alternative or complementary strategies to antibiotic treatment, particularly in cases where antibiotic treatment is inappropriate or ineffective. In the case of *C. difficile*, initial infection is typically caused by the gastrointestinal microbial dysbiosis induced from antibiotic use, and rising recurrence rates suggest that alternative therapies that do not alter the host microbiome are required. In the case of botulism, antibiotics are typically not used for treatment as infection results from intake of the toxin itself, not the bacterium. Coupled with the demonstrated clean safety profile of the parent compound ebselen in human clinical trials, selenides present an opportunity to develop broad anti-virulence agents to target multiple pathogenic exotoxins that could be rapidly advanced to the clinic.

METHODS

Protein expression and purification.

Bacterial cultures were grown at 37 °C with shaking unless otherwise specified. The gene encoding BoNT/A LC residues 1 – 425 were cloned into the pTrcHisA vector containing an N-terminal His_6 tag. Site-directed mutagenesis to create BoNT/A LC constructs C165A, C134A, C165A/C134A, and E262Q was performed with Phusion from the wild-type pTrcHisA-BoNT/A LC 1–425 vector. Constructs were transformed into chemically competent *E. coli* BL21(DE3) cells (New England Biolabs) for expression. Saturated overnight cultures grown under ampicillin selection were diluted 1:100 into 2xYT medium. Expression was induced at OD_{600} of approximately 0.6 by addition of 1 mM β-d-1-thiogalactopyranoside (IPTG) and expression was allowed to proceed overnight at 20 °C.

CS25R substrate containing fluorescent proteins Clover and mRuby2 flanking SNAP-25 residues 141–206 was cloned into the pBAD vector containing an N-terminal His_6 tag and transformed into chemically competent *E.* coli DH5α cells. A saturated overnight culture grown under ampicillin selection was diluted 1:100 into 2xYT medium and grown to OD_{600} of approximately 0.6. Expression was induced by addition of 0.2% (w/v) final concentration arabinose and expression was allowed to proceed for 4 hours at 37 °C.

For all purifications, cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0; 500 mM NaCl, 5 mM imidazole), treated with 1 mg ml^{-1} lysozyme for 30 minutes on ice, and membranes were disrupted via sonication on ice. Lysates were clarified by centrifugation at 16,500 rpm for 30 minutes. N-terminally tagged constructs were purified with Ni^{2+} metal affinity chromatography. Purified protein was eluted with high imidazole buffer (250 mM) and buffer exchanged to storage buffer (50 mM HEPES, pH 7.1, 10% (v/v) glycerol) on a PD-10 column (GE Life Sciences). Purified proteins were stored at –80 °C.

5-IAF competition assay optimization.

BoNT/A LC C134A was diluted to 1 μM in 14 μL of 5-IAF assay buffer (50 mM HEPES, pH 7.1, 10 μM $ZnCl_2$, 1% (v/v) NP40) and pre-treated with DMSO or different concentrations of NEM (30X stock in DMSO) at 37 °C for 10, 30, or 120 minutes. Samples were then labeled with 1 μM 5-IAF (30X stock in DMSO) at 37 °C for 5, 10, or 30 minutes.

5-IAF competition assay with BoNT/A LC constructs.

BoNT/A LC wild-type, C134A, C165A, or C134A/C165A was diluted to 1 μM in 14 μL of 5-IAF assay buffer and pre-treated with DMSO or different concentrations of NEM (30X stock in DMSO) at 37 °C for 30 minutes. Samples were then labeled with 1 μM 5-IAF (30X stock in DMSO) at 37 °C for 30 minutes.

5-IAF competition screen.

BoNT/A LC C134A was diluted to 1 μM in 102.9 μL of 5-IAF assay buffer and pre-treated with DMSO, NEM or 10 μM final concentration of cysteine-reactive compound (100X stock in DMSO) at 37 °C for 30 minutes in a 96-well format. Samples were then labeled with 1 μM 5-IAF (100X stock in DMSO) at 37 °C 30 minutes.

Analysis of 5-IAF labeling.

For all experiments, reactions were quenched with 4X SDS sample buffer and boiled at 95 °C. Samples were resolved by SDS-PAGE, scanned for fluorescence with a typhoon imager in the fluorescein channel, and then stained with Coomassie to ensure equal loading. Experiments were performed in triplicate and fluorescent signals were quantified using Alpha Imager software. Fluorescent signal for each band was normalized to Coomassie intensity to control for loading.

Mass spectrometry with BoNT/A LC constructs and ebselen.

BoNT/A LC wild type, C134A, and C134A/C165A were diluted to 10 μM in BoNT activity buffer (50 mM HEPES, pH 7.1, 10 μM $ZnCl_2$) and incubated with 100 μM ebselen (from 10 mM stock in DMSO) or 1% (v/v) DMSO for 15 minutes at 37 °C. Samples were analyzed on a Waters 2795 HPLC system with dual wavelength UV detector, and ZQ single quadrupole MS with electrospray ionization source. Spectra were deconvoluted using MassLynx software to yield reported masses.

Time-dependence of BoNT/A LC constructs in gel-based BoNT/A LC activity assay with CS25R.

BoNT/A LC wild type, C165A, C134A, or E262Q was diluted to 10 nM in 10 μM of BoNT activity assay buffer. CS25R substrate was diluted to 3.6 μM in BoNT activity assay buffer. At time 0, 5 μL of CS25R was added to BoNT, for a final concentration of 1.2 μM substrate. Reactions were incubated at 37 °C and quenched at time 0, 5, 10, 30 or 60 minutes with 4X SDS sample buffer and boiled. Samples were resolved by SDS-PAGE and stained with Coomassie to visualize residual full-length CS25R substrate.

Testing compounds as BoNT inhibitors in gel-based BoNT/A LC activity assay with CS25R.

BoNT/A LC wild-type or C134A/C165A was diluted to 10 nM in 9.5 μL of BoNT activity assay buffer and pre-treated with different concentrations of compound (30X stock in DMSO) or DMSO control for 15 minutes at 37 °C. To measure residual enzyme activity, 5 μL of CS25R substrate diluted to 3.6 μM in BoNT activity assay buffer was added to the reaction for a final concentration of 1.2 μM substrate and incubated for 1 hour at 37 °C.

Reactions were quenched with 4X SDS sample buffer and boiled. Samples were resolved by SDS-PAGE and stained with Coomassie to visualize residual full-length CS25R substrate.

FRET-based BoNT/A LC activity assay with CS25R.

BoNT/A wild-type was diluted to 12.66 nM in 39.5 μL BoNT activity assay buffer and added to a 384-well black-sided, black-bottom plate. BoNT was pre-treated with 0.5 μL compound at the indicated concentrations (100X in DMSO stock) or DMSO control and incubated for 15 minutes at 37 °C. All at once, 10 μL of 250 nM CS25R substrate was added to each well, and the plate was immediately read on a plate reader with excitation wavelength 485 nm and emission wavelengths of 520 nm (Clover) and 600 nm (mRuby2) every minute for 1 hour on 'sweep' mode. Assays were run on a Cytation3 plate reader. Residual BoNT activity was measured by plotting the ratio between the FRET donor signal (Clover) over the FRET acceptor signal (mRuby2). Raw slope values were calculated as the slope in donor/acceptor FRET ratio vs. time for the first 10 minutes of the experiment. Percent activity was calculated by normalizing the raw slope values to fall between 0% (the average slope for wells without BoNT/A) and 100% (the average slope for BoNT/A treated with 1% DMSO).

Gel-based BoNT/A LC activity assay with CS25R before or after dialysis of enzyme-inhibitor complex.

BoNT/A LC was diluted to 500 nM in BoNT activity assay buffer and pre-treated with 500 μM of 2,4-dichlorocinnamic acid hydroxamate or ebselen and incubated for 30 minutes at 37 °C. The enzyme-inhibitor mixture was then dialyzed in Mini Slide-A-Lyzer casettes (Thermo Scientific) into 500 ml (10,000x volume) of BoNT activity assay buffer for 1 hour at 4 °C. To measure residual enzyme activity, CS25R substrate was added to the reaction for a final concentration of 1.2 μM substrate and incubated for 1 hour at 37 °C. Reactions were quenched with 4X SDS sample buffer and boiled. Samples were resolved by SDS-PAGE and stained with Coomassie to visualize residual full-length CS25R substrate.

Kinetic analysis of BoNT inhibition.

Ebselen was assumed to inhibit BoNT/A LC via a two-step mechanism: reversible binding followed by an irreversible reaction step. FRET acceptor/donor ratio was fit to Equation 1 to yield k_{obs} at each ebselen concentration. K_I and k_{inact} were estimated by nonlinear fitting of Equation 2 to k_{obs} as a function of inhibitor concentration.

$$p(t) = \frac{v_0}{k_{obs}}\left(1 - e^{-kobs \cdot t}\right) \quad \text{Equation 1.}$$

P, product concentration; t, time; v_0, initial rate; k_{obs}, observed rate of inhibition.

$$k_{obs} = \frac{k_{inact}[I]}{k_I + [I]} \quad \text{Equation 2.}$$

[I], inhibitor concentration; K_I, equilibrium constant for reversible binding; k_{inact}, rate of irreversible reaction.

Neuron culture.

Primary rat cortical neurons were prepared from E19 embryos (Sprague Dawley strain, pregnant females were purchased from Charles River) using papain dissociation kit (Worthington Biochemical). Neurons were harvested on Poly-D-lysine (PDL) coated 24-well plate (0.25×10^6 cells/well) and cultured in Neurobasal medium (Thermo Fisher Scientific) with 1 x B27 (Thermo Fisher Scientific) and 0.5 % (v/v) fetal bovine serum.

Toxicity assay with selenide inhibitors on rat primary cultured neurons.

Rat cortical neurons were plated into PDL-coated 96-well plates (0.25×10^5 cells/well). Neurons were then incubated with the indicated concentration of inhibitor in 100 μL of medium for three days. Toxicity of the neurons was assessed daily via morphologic changes and scored from mild to severe neuronal toxicity.

BoNT/A light chain activity assay in brain detergent extract.

2 μM of BoNT/A LC (2X final concentration) was pre-incubated with 2X the indicated final concentration of each compound at room temperature for 30 min in 20 μL of Tris buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). To each BoNT/A LC and selenide compound mixture, 20 μL of approximately 2 mg ml^{-1} rat brain detergent extract (prepared as in Zhang, *et al*[23]) was then added and incubated at 37 °C for 1 hour. Samples were resolved via SDS-PAGE, with full-length and cleaved SNAP-25 substrate measured via western blot with mouse monoclonal antibodies for SNAP-25 (71.1, 1:2,000 dilution, Synaptic Systems) and actin (AC-15, 1:1,000, Sigma) to measure loading.

Full-length BoNT/A activity assay with selenide inhibitors in rat primary cultured neurons.

100 pM of full-length BoNT/A (META biology, Inc.) was pre-treated with 10X the indicated final concentrations of each compound at 37 °C for 30 minutes in 30 μL medium. Full-length BoNT/A and selenide mixture was mixed with 270 μL medium and added to primary rat neuronal culture and incubated at 37 °C for 14 hours. Neurons were harvested by lysis buffer (1% (v/v) Triton X-100, 0.05% (w/v) SDS, protease inhibitor cocktail). Lysates were centrifuged for 10 min at maximum speed at 4 °C. Supernatants were resolved by SDS-PAGE and detected SNAP-25 and actin by immunoblotting.

Chemical Synthesis.

The MG series of selenides was obtained from Mirosław Giurg and coworkers, with synthetic methods reported.[24–26] The synthesis of SL ebselen analogues was performed with KSeCN as described previously.[27] Details of the synthesis for novel compounds are described in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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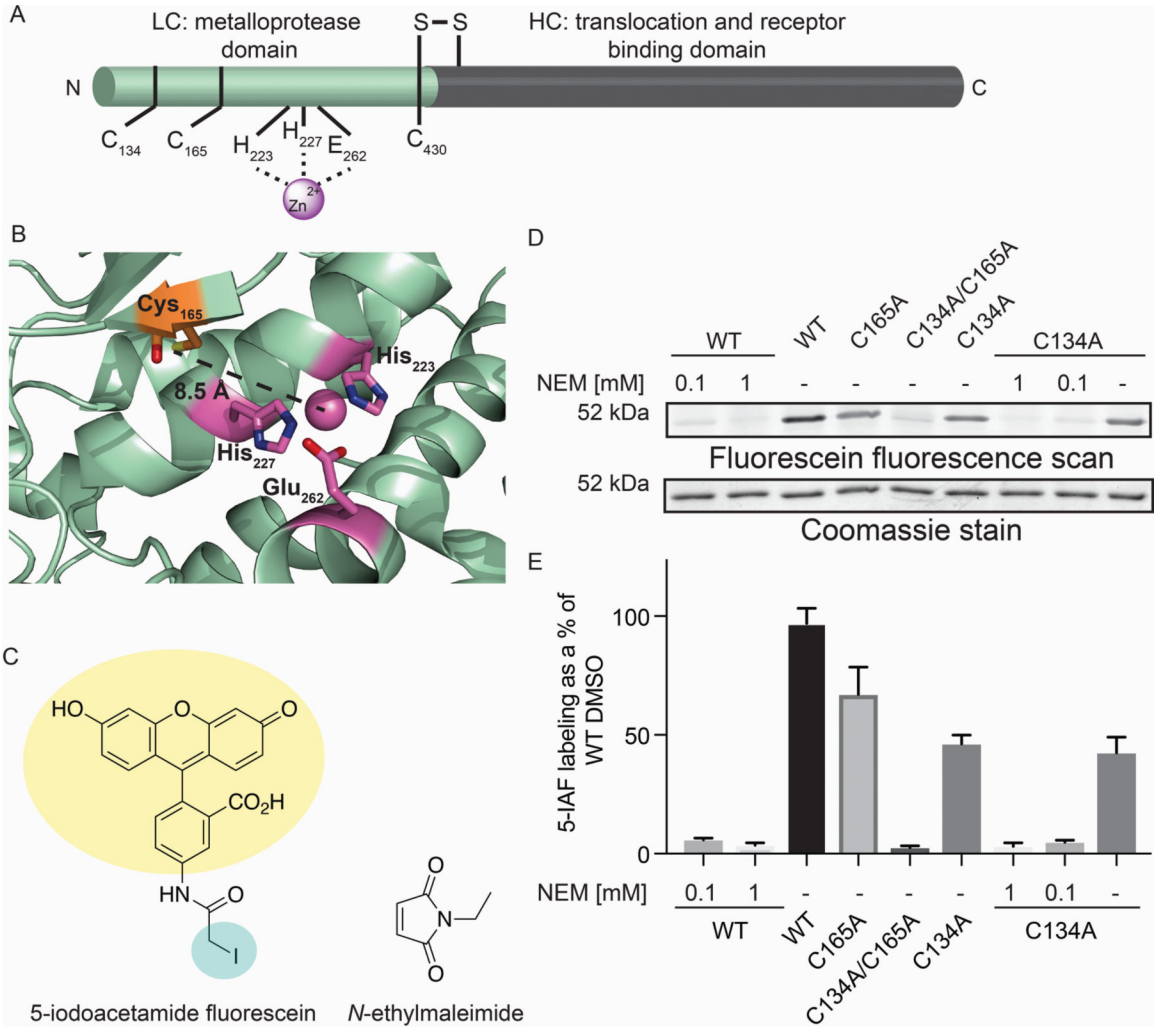
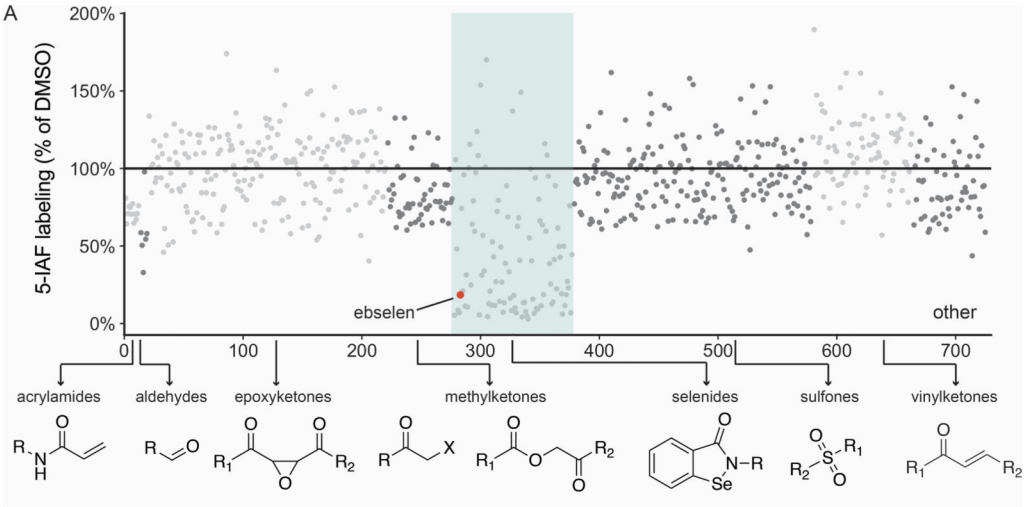
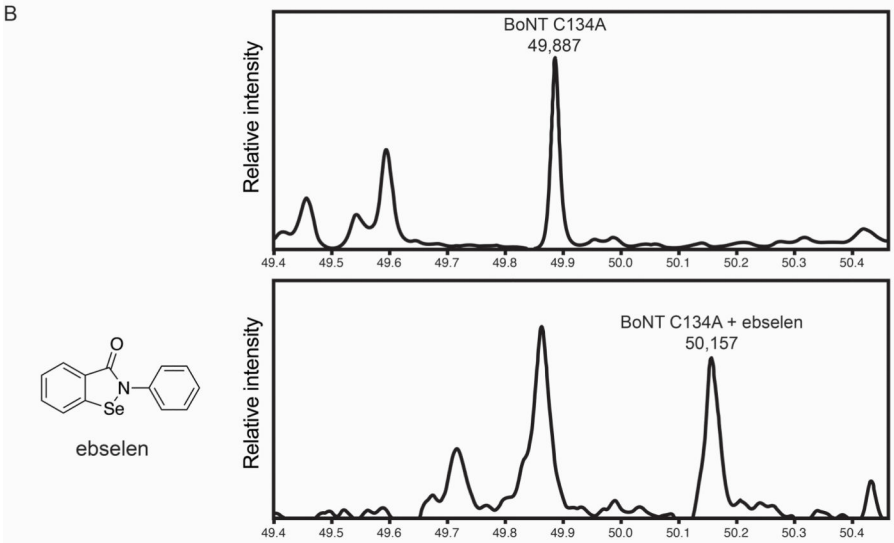


Figure 1.

Development of a competition screen with the cysteine-reactive compound 5-IAF. A. Schematic of the full-length botulinum neurotoxin A (BoNT/A) consisting of a metalloprotease domain (LC) and a translocation and receptor binding domain (HC). Highlighted on the LC are three cysteine residues and the triad that coordinates the zinc ion. Magenta sphere: Zn^{2+} ion. B. Crystal structure of BoNT/A LC (PBD 4EJ5) highlighting the approximate distance of 8.5 Å between the active-site adjacent cysteine (C165, orange) and the catalytic zinc (magenta). Magenta residues: zinc-coordinating triad. C. Left: structure of the cysteine-reactive fluorescent compound, 5-iodoacetamide fluorescein (5-IAF). The fluorescein fluorophore is highlighted in yellow and the iodoacetamide warhead is highlighted in blue. Right: structure of the generic cysteine-reactive compound *N*-ethylmaleimide (NEM). D. Gel-based competition assay for covalent modification of cysteines. WT, C165A, C134A, or C134A/C165A BoNT/A LC constructs were pre-incubated with different concentrations of NEM or DMSO, with unmodified cysteines subsequently fluorescently labeled upon addition of 5-IAF. Samples were resolved with SDS-PAGE, visualized with a fluorescent scanner in the fluorescein channel (top), and stained with Coomassie (bottom) as a loading control. E. Quantification of (D) showing

residual 5-IAF florescent signal, normalized to Coomassie loading control and plotted as a percentage of 5-IAF labeling with WT DMSO control (lane 3). Error bars indicate standard deviation measured from three independent experiments.





C

Protein	Modification	Expected (Da)	Observed (Da)	Mass error (Da) [ppm]
WT	-	49,776	49,777	1 [20]
	1 ebselen	50,050	50,045	5 [100]
	2 ebselen	50,234	50,340	16 [317]
C134A	-	49,873	49,887	14 [281]
	1 ebselen	50,147	50,157	10 [200]
C134A/C165A	-	49,841	49,851	10 [200]

Figure 2: A competition screen with 5-IAF identifies selenides as the preferred covalent modifiers of BoNT/A LC C165.

A. Dot plot of screening results of the gel-based competition assay with cysteine-reactive libraries and 5-IAF. BoNT/A LC C134A was pre-incubated with 10 μM of compound, NEM or DMSO, with unmodified cysteines subsequently fluorescently labeled upon addition of 5-IAF. Dotplot quantification displays residual 5-IAF florescent signal, normalized to Coomassie loading control and plotted as a percentage of 5-IAF labeling with DMSO control. Cysteine-reactive warheads screened are highlighted with compounds arranged by warhead, with alternating gray and dark gray circles indicating different warhead groups.

Compounds containing a selenide warhead, with ebselen as the parent compound (red circle), are highlighted in the blue box. B. Left: Structure of ebselen (2-phenyl-1,2-benzoselenazol-3-one), parent selenide compound. Right: Deconvoluted mass spectra of BoNT/A LC C134A (top) and BoNT/A LC C134A pre-incubated with ebselen (bottom). The identified mass for the LC or LC plus ebselen is indicated. C. Table summary of mass spectrometry experiments with BoNT/A LC constructs and ebselen shown in (B) and Figure S3.

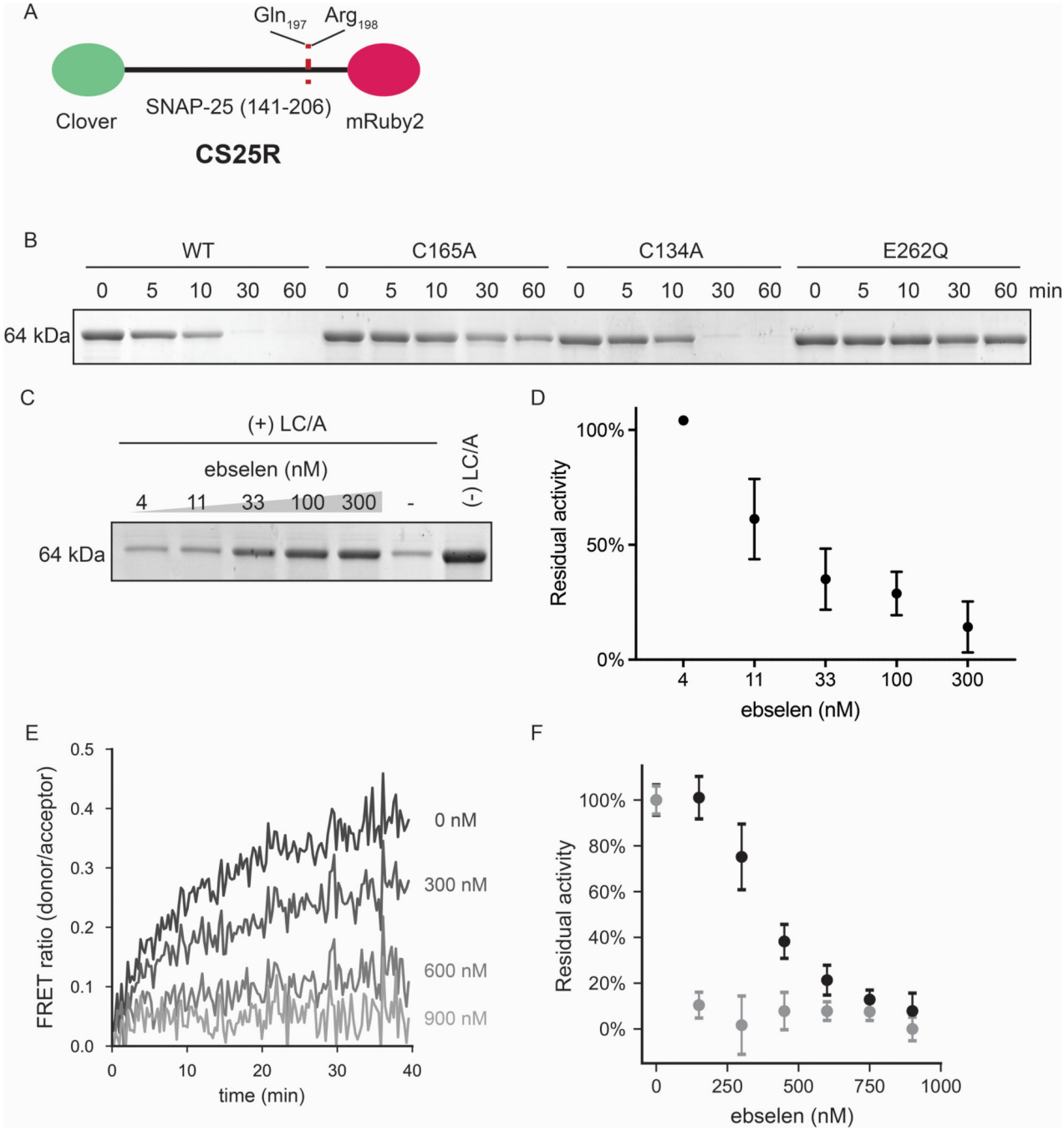


Figure 3: Parent selenide ebselen inhibits BoNT/A LC protease activity *in vitro*.

A. Construct of the fusion protein, CS25R, used in activity assays. The substrate contains 66 amino acids of the physiologic substrate, SNAP-25, flanked by the fluorescent proteins Clover and mRuby2. The cleavage site between glutamine and arginine is highlighted. B. Gel-based activity assay with BoNT/A LC constructs. Wild-type, C165A, C134A, or E262Q was incubated with CS25R substrate for the indicated time points. Samples were resolved by SDS-PAGE and stained with Coomassie. Residual LC activity was measured by cleavage of the CS25R substrate resulting in loss of full-length CS25R upon visualization with

Coomassie stain. C. Gel-based activity assay shows dose-dependent protection of CS25R from BoNT/A LC cleavage by ebselen. BoNT/A LC was pretreated with different concentrations of ebselen, then CS25R substrate was added. Samples were analyzed as in (B). D. Percentage residual LC activity quantified from the gel-based assay. Results are displayed for three independent experiments (4 nM, n=1) ± SEM. E. A FRET assay using the CS25R fusion protein measures change in FRET ratio as SNAP-25 substrate is cleaved. Different concentrations of ebselen and substrate were added concurrently (no pre-treatment) to BoNT/A LC WT. FRET signal was measured as the ratio of donor (Clover; 520 nm) to acceptor (mRuby2; 600 nm) emission upon excitation at 485 nm. F. Percentage residual BoNT/A LC activity measured from FRET assay as in (E) with different pre-treatment times of BoNT/A LC with ebselen (black, no pre-treatment; gray, 30 minutes pre-treatment). Results are displayed for three independent experiments ± standard deviation.

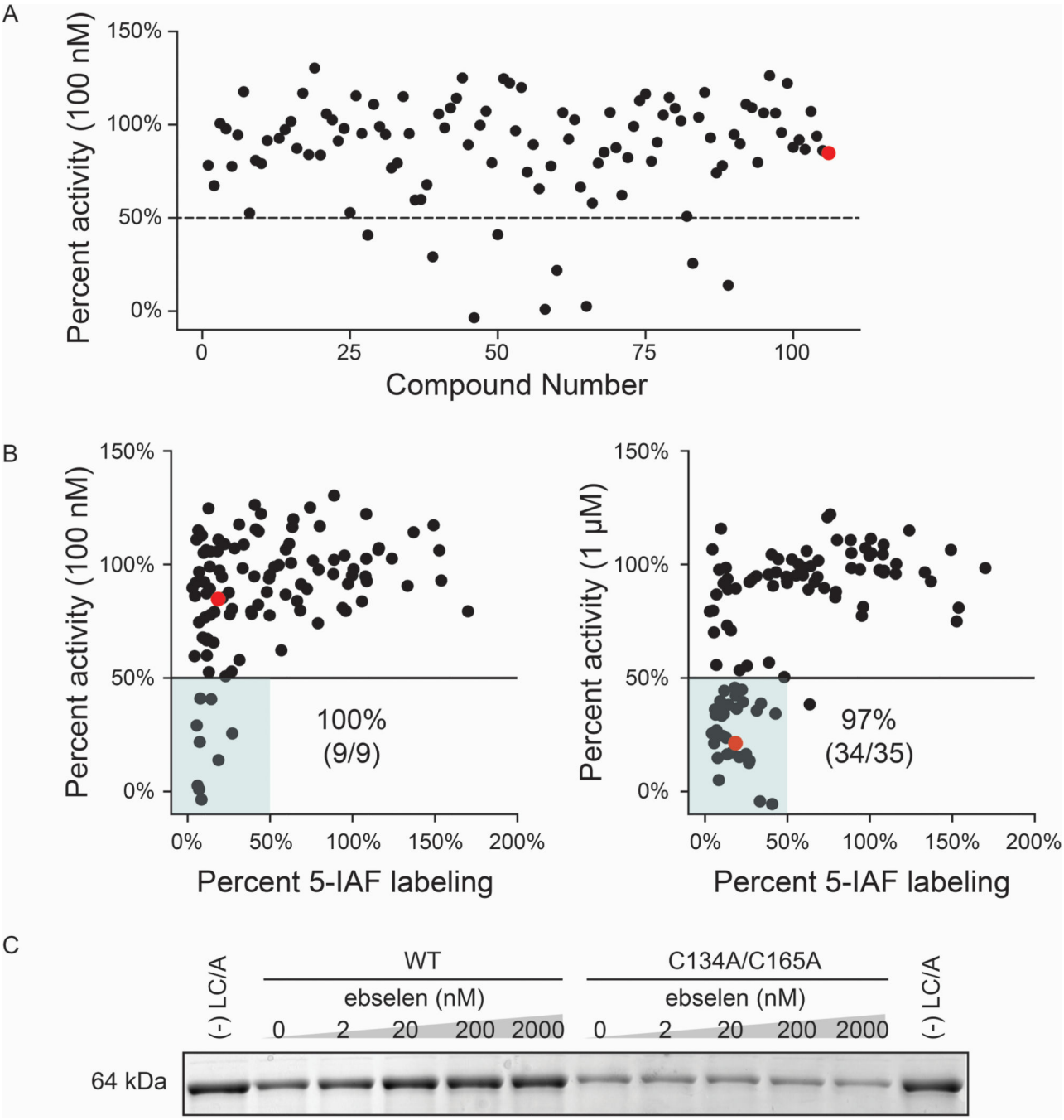
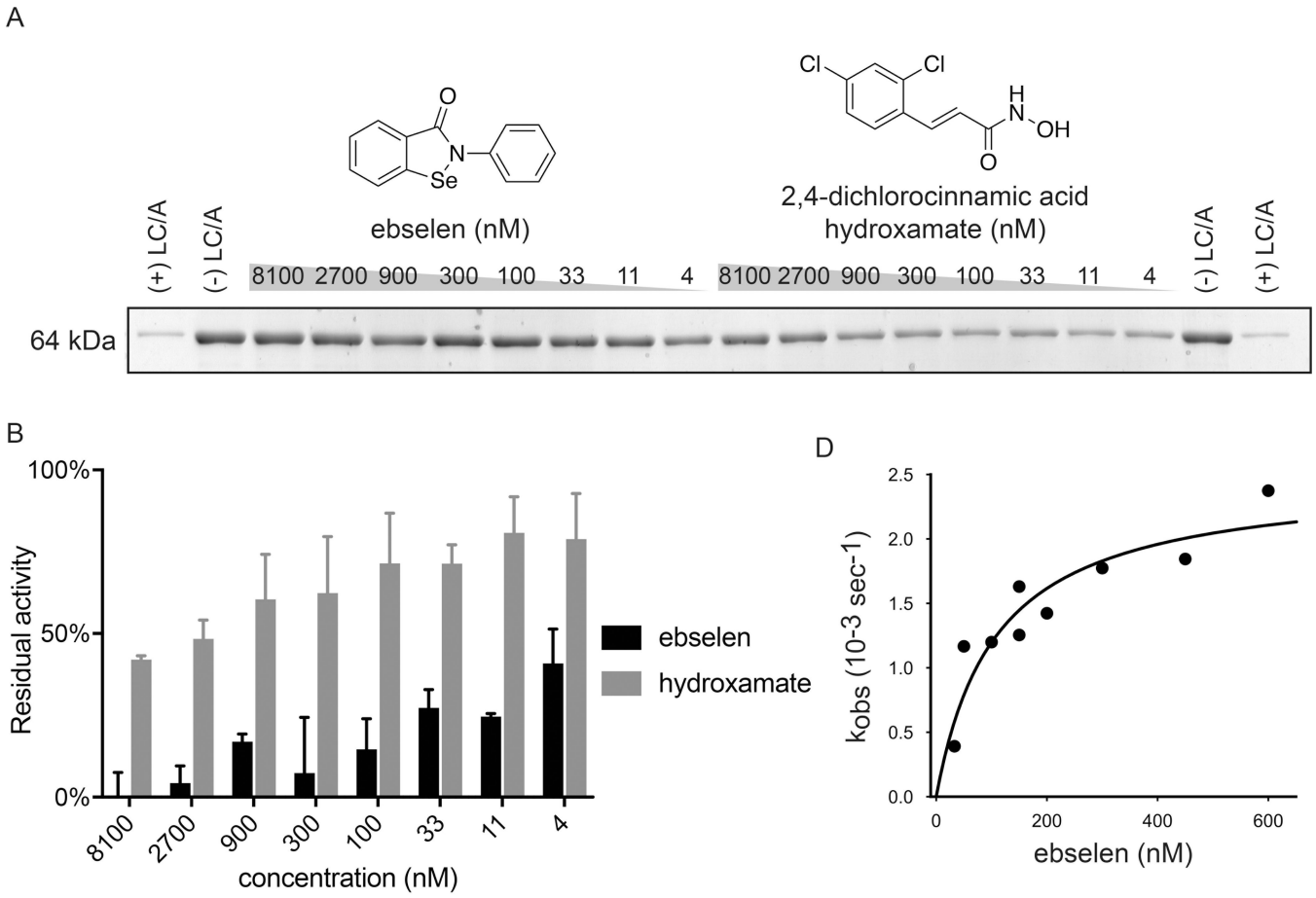


Figure 4: Covalent modification at C165 correlates with BoNT/A LC inhibition and is cysteine-dependent.

A. Results of FRET assay with selenide compounds screened at 100 nM. BoNT/A LC WT was pre-incubated with 100 nM of compound, then substrate was added. FRET signal was measured as the ratio of donor (Clover; 520 nm) to acceptor (mRuby2; 600 nm) emission upon excitation at 485 nm. Activity was measured as the slope of the linear region of the FRET progress curve and was normalized to DMSO-treated (100%) and toxin-free (0%) controls. Assays were performed in triplicate. The red circle indicates the parent compound, ebselen. B. Comparisons of percent activity from the FRET screen (at 100 nM (left) and 1

μM (right)) versus 5-IAF labeling for each compound. Red circles indicate the parent compound, ebselen. Percentage within each plot represents the number of hits in the FRET assay (denominator) also designated as hits in the 5-IAF competition assay (numerator). C. Inhibition of protease activity by the parent selenide, ebselen. BoNT/A LC wild-type or C134A/C165A was pre-incubated with different concentrations of ebselen before adding CS25R substrate. Samples were resolved by SDS-PAGE and stained with Coomassie. Residual LC activity was measured by cleavage of the CS25R substrate resulting in loss of full-length CS25R upon visualization with Coomassie stain.



E

Kinetic parameters	ebselen
k_{inact} (sec^{-1})	2.5×10^{-3}
K_I (nM)	110
k_{inact}/K_I (M^{-1} sec^{-1})	23,000

Figure 5: Covalent modifiers of the BoNT/A light chain permanently inactivate metalloprotease activity.

A. Gel-based activity assay with different concentrations of the previously published compound, 2,4-dichlorocinnamic acid hydroxamate (hydroxamate) or ebselen. BoNT/A LC was pre-treated with different concentrations of the hydroxamate or ebselen, then CS25R was added. Samples were resolved by SDS-PAGE and stained with Coomassie. Residual LC activity was measured by cleavage of the CS25R substrate resulting in loss of full-length CS25R upon visualization with Coomassie stain. B. Percentage residual activity of BoNT/A LC quantification from gel in (A). Results displayed as the mean of three independent experiments ± SEM. C. Gel-based activity assay with BoNT/A LC and CS25R before and

after dialysis. BoNT/A LC was pretreated with 1000x concentration of hydroxamate or ebselen, then the enyme-inhibitor mixture was dialyzed to remove unbound or reversibly bound inhibitor. The enzyme-inhibitor complex was then incubated with CS25R substrate, with full-length CS25R visualized via Coomassie stain. D. Determination of irreversible inhibitor kinetic parameters with ebselen. Plot of k_{obs} versus ebselen concentration. E. Summary of kinetic parameters with ebselen determined from data shown in (D).

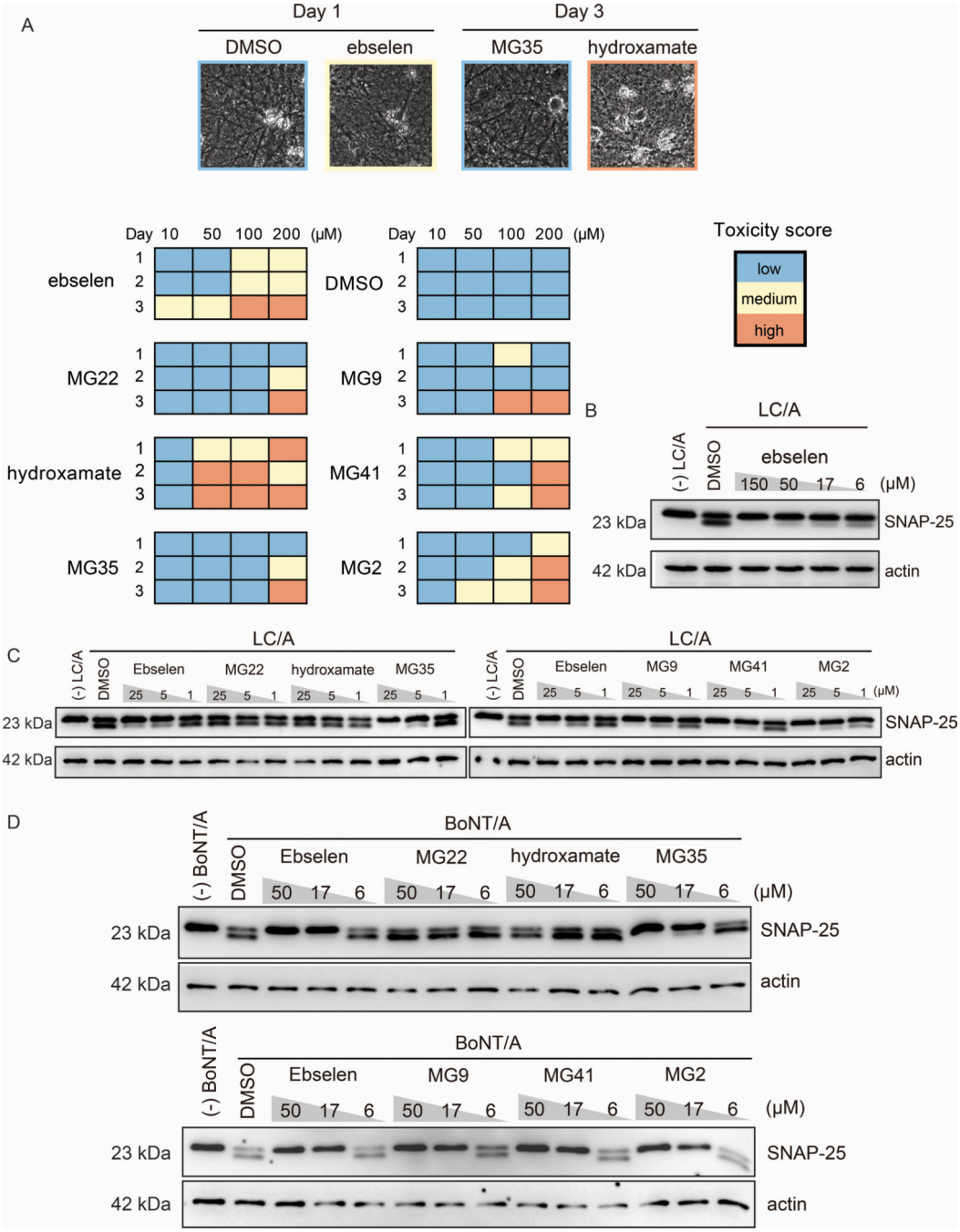


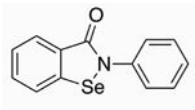
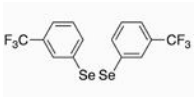
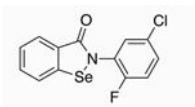
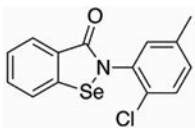
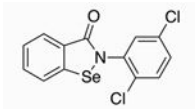
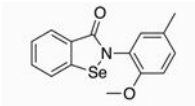
Figure 6: Selenides inhibit full-length BoNT/A in neuronal assays.

A. Toxicity assay in primary neuronal culture. Top: Representative images of morphological changes to neurons used to designate toxicity scores. Bottom: Toxicity scores for top non-toxic compound and controls at indicated concentrations over three days. B. Dose-dependent protection of ebselen in brain detergent extract. Ebselen was incubated with BoNT/A LC for 30 minutes at room temperature, then 40 μg brain detergent extract was added to the protease-selenide mixture. Reactions were incubated at 37 °C for 30 minutes, then samples were resolved by SDS-PAGE and analyzed via western blot for full-length SNAP-25 (top

band) and cleaved SNAP-25 (lower band). Actin was used as a loading control. C. Dose-dependent protection of selenide compounds in brain detergent extract. Samples were treated with the indicated concentrations of compound as in (B) and analyzed via western blot as in (B). D. Inhibition of full-length BoNT/A in primary neuronal culture. Full-length BoNT/A was pre-treated with the indicated concentrations of compounds for 30 minutes at 37 °C. The BoNT/A-compound mixture was then added to primary neuronal culture for 14 hours. Lysates were prepared, resolved with SDS-PAGE, and analyzed via western blot as in (B).

Table 1:

Selenide compounds tested in primary neuron culture

Compound	Structure	%5-IAF labeling	% residual BoNT/A activity (1μM)	% residual BoNT/A activity (100nM)
ebselen		18%	21%	85%
MG22		108%	109%	103%
MG35		9%	40%	68%
MG9		13%	24%	53%
MG41		8%	15%	41%
MG2		12%	38%	67%