High-Throughput Multiplex Flow Cytometry Screening for Botulinum Neurotoxin Type A Light Chain Protease Inhibitors

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ABSTRACT

Given their medical importance, proteases have been studied by diverse approaches and screened for small molecule protease inhibitors. Here, we present a multiplexed microsphere-based protease assay that uses high-throughput flow cytometry to screen for inhibitors of the light chain protease of botulinum neurotoxin type A (BoNTALC). Our assay uses a full-length substrate and several deletion mutants screened in parallel to identify small molecule inhibitors. The use of multiplex flow cytometry has the advantage of using full-length substrates, which contain already identified distal-binding elements for the BoNTALC, and could lead to a new class of BoNTALC inhibitors. In this study, we have screened 880 off patent drugs and bioavailable compounds to identify ebselen as an in vitro inhibitor of BoNTALC. This discovery demonstrates the validity of our microsphere-based approach and illustrates its potential for high-throughput screening for inhibitors of proteases in general.

INTRODUCTION

Proteases regulate many biological pathways, including blood clotting and immune system activation,¹ bacterial toxins,² metastasis,³ viral life cycles,⁴ among many others. For these reasons, a large number of proteases are currently under investigation as pharmaceutical targets.² Within the larger set of proteases, pharmaceutical development for the botulinum neurotoxin type A light chain (BoNTALC) is of interest due its role in natural disease,⁵ its potential role in biothreat scenarios, and increasing use as a pharmaceutical,⁶ which increases the potential for both accidental and intentional misuse.⁷

The BoNTALC protease, which is delivered to the cell via the toxin heavy chain, is a Zn²⁺ metalloprotease that specifically cleaves synaptosome-associated protein of 25 kDa (SNAP-25), causing paralysis and death.⁸ SNAP-25 contains potential conserved sites of protease interaction, termed the SNARE motif at 4 positions. Three N-terminal SNARE motifs, S1 (residues 21–31), S2 (residues 35–45), S3 (residues 49–59) are present, and the S4 SNARE motif (residues 145–155) is located near the cleavage site.⁹ Activity assays strongly suggest that S4 contains a preferred SNARE interaction site and is required for specific BoNTALC activity.^{10,11} The importance of the S4 site was confirmed via identification of the α -exosite in the co-crystal structure between SNAP-25 and BoNTALC,¹² which contains many contacts between the 2 proteins and overlaps the S4 SNARE motif. These observations, taken together, have led to a generally accepted mechanism: BoNTALC first binds to the α -exosite of SNAP-25, corresponding to the S4 site, which creates a conformational change in BoNTALC, and leads to proteolytic cleavage of SNAP-25.^{12,13} Furthermore, the

ABBREVIATIONS: BoNTALC, botulinum neurotoxin type A light chain; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; GFP/EGFP, green fluorescent protein/enhanced green fluorescent protein; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl- β -D-thiogalactopyranoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTI, Photon Technology International; SNAP, 25- synaptosome-associated protein of 25 kDa; TB, terrific broth.

co-crystal structure indicates that BoNTALC has close contacts at many positions in the substrate beyond the α -exosite.¹² Thus, use of the full-length SNAP-25 substrates, in the context of the proposed mechanism, makes its use highly desirable in screening assays.

Despite the desirability of full-length substrates, most BoNTALC screening assays use small peptides and fluorescence resonance energy transfer (FRET) to monitor cleavage.^{14,15} The potential for discovery of inhibitors that interfere with BoNTALC-SNAP-25 interaction at the α -exosite has been suggested but have yet to be discovered.^{9,12,13} Such inhibitors could be highly desirable as they would not likely be competitive inhibitors, which could reduce their potential for toxicity as BoNTALC contains the highly conserved Zn²⁺ metalloprotease active site found in many human proteases.^{16,17} Alternatively, cleavage of fluorescently labeled proteins from a surface offers the ability to use full-length protease substrates. Among the several assays of this type, microsphere-based protease assays are particularly compelling as protease activity can be measured via the loss of a fluorescent protein from the surface of the microsphere, which is performed very simply on a flow cytometer due to its inherent ability to discriminate fluorescent molecules bound to the surface vs. those in solution.^{18,19} Flow cytometry also enables simultaneous analysis of multiple substrates in the same sample via use of distinct microsphere populations for each substrate, each distinguished by differing amounts of internal fluorescence intensity.

In this work, we have developed a set of fluorescent SNAP-25 substrates that are attached to a multiplex set of microspheres and measured BoNTALC activity by loss of microsphere-associated fluorescence as monitored by flow cytometry. We have validated the use of this assay for high-throughput screening (HTS) of protease inhibitors by implementing it on the HyperCyt® high-throughput flow cytometry screening system^{18,20,21} to rapidly screen a small molecule library of chemical compounds.

MATERIALS AND METHODS

Reagents

Hepes hemisodium salt, sodium chloride, phosphate-buffered saline (PBS) carbenicillin, chloramphenicol, biotin, dithiothreitol (DTT), isopropyl- β -D-thiogalactopyranoside (IPTG), Tris base, Tween 20, bovine serum albumin (BSA), and ebselen were obtained from Sigma-Aldrich corporation (St. Louis, MO). BoNTALC and the SnapTide FRET peptide were obtained from List Biological Laboratories (Campbell, CA). SoftLink™ streptavidin resin was obtained from Promega Corporation (Madison, WI). Streptavidin-coated pink particle kit was obtained from SpheroTech corporation (Lake Forest, IL). Amicon Ultra-15 filters were purchased

from Millipore (Billerica, MA). Terrific broth (TB) was purchased from Fisher scientific (Pittsburg, PA). Restriction enzymes and ligase were obtained from New England Biolabs (Ipswich, MA). DNA oligonucleotides were synthesized by IDT (Coralville, IA) and Operon (Huntsville, AL). The 96-well plates were obtained from ISC BioExpress (Kaysville, UT). QIAprep spin miniprep kit was purchased from Qiagen (Hilden, Germany). The 8-mL glass econo-columns were obtained from BIO-RAD Laboratories (Hercules, CA).

Construction of Biotinylated Substrate Plasmids

Protease substrate plasmids were based on the Promega PinPoint biotinylation tag vectors with enhanced green fluorescent protein (EGFP) cloned at the C terminus of the open reading frame as described previously.¹⁸ The negative control plasmids Xa sub and lethal factor (LF) substrate were created as described previously.¹⁸ The SNAP-25 green fluorescent protein (GFP) plasmid was created by restriction digest of the Xa sub-plasmid with (*Bam*HI and *Sac*I) and ligation of a SNAP-25 polymerase chain reaction (PCR) product from a SNAP-25 clone plasmid obtained from Open Biosystems (Huntsville, AL) using the primers CCACCGAGCTCATGGCCGAAGACGCAGACAT and GTGGCGGATCCGCCCACTTCCCAGCA and digested with *Sac*I and *Bam*HI, into the Xa sub-plasmid between the biotinylation tag and EGFP. The binding site deletion plasmids SNAP-25 Δ S1-S3 and SNAP-25 Δ S4 were created by whole plasmid PCR of the SNAP-25 full-length plasmid with PCR primers flanking the binding sites of interest containing an *Apa*I restriction site and ligated together. SNAP-25 Δ S1-S3 was created using the primers GAGCTAGGGCCCGTCGAAGAAGGCATGAACC and CCAATCGGGCCCTGGTCAGCCCTTCGCTGCA. SNAP-25 Δ S4 was created using the primers GGGCCCATCATCGGGAACCTCCGT and GGGCCCATTTCTCGGGCATCATTTG.

The SNAP-25 Δ S1-S4 plasmid was created by using the SNAP-25 Δ S1-S3 plasmid and the primers GACTACACGCGTGGCATCATCGGGAACCTCCGTCACATGGCCCTGGA and GACTCAACGCGTATTTCTCGGGCATCATTTGTTACCCTGCGGATGAAGCCG, digesting with *Mlu*I and ligating the plasmid ends together. The LF15 optimized lethal factor cleavage sequence was cloned by digesting the Xa sub-plasmid with *Sac*I and *Hind*III and using the 5' phosphorylated DNA oligonucleotides of sequences CAGAAGAAAGAAGTTTATCCATATCCAATGGAAACCATTGCTGGTCA and AGCTTGACAGCAATGGTTTCCATTGGATATGGATAAACCTTCTTCTTCTGGAGCT. Plasmid ligations were transformed into calcium competent SCS1 *Escherichia Coli*. minipreps were done with a Qiagen QIAprep miniprep kit and were sequenced to confirm correct sequences.

Expression and Purification of Biotinylated Protease Substrates

Calcium competent BL21 (DE3) pLys S *E. coli*, transformed with plasmids of interest, were grown overnight in 3 mL of TB media containing 50 µg/mL of carbenicillin and 34 µg/mL chloramphenicol then transferred to 200 mL of TB containing 50 µg/mL of carbenicillin and 34 µg/mL chloramphenicol and 40 µM biotin and grown at 37°C to an optical density at wavelength 600 between 0.6 and 0.8. Cultures were then induced using 100 µM of (IPTG) and grown at 30°C overnight in a shaking incubator. Cells were spun down at 10,000g for 10 min on a Beckman Avanti J-301 centrifuge at 4°C, suspended in lysis buffer (50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, pH 8.3), and frozen at -20°C.

Frozen cells were thawed on ice and sonicated on Branson Sonifier 250 model sonicator for 10 min on pulse setting, then centrifuged at 13,000g for 30 min at 4°C. Soluble fractions were then poured into fresh centrifuge tubes and centrifuged at 13,000g for 30 min at 4°C. The supernatant was then pumped with a peristaltic pump over a 8-mL glass Bio-Rad econo-column containing 5 mL of SoftLink™ streptavidin resin at a flow rate of 1 mL/min at 12°C. The column was washed with 200 mL of PBS containing 1 mM DTT, to prevent cross-linking of cysteine-rich molecules, at a rate of 1 mL/min. The column was then filled with 6 mL of PBS with 1 mM DTT and 5 mM biotin and let set for 30 min before elution with the same buffer. Elution fractions were collected and combined into a 12–15 mL pool. Four-milliliter fractions of this pool were centrifuged in an Amicon Ultra-15 30,000 molecular weight cutoff filter for 10 min at 4,000g at 4°C and condensed into 500 µL fractions. The remaining purified protein from the 12–15 mL pool was added and this was repeated until the entire elution pool was in a volume of 500 µL to 1 mL. Four milliliter of PBS with 1 mM DTT was added to the Amicon Ultra-15 Mw 30,000 cut-off filter and centrifugation was repeated to remove free biotin; this process was repeated 20 times to remove as much free biotin as possible. One to two milliliters of purified protein was then dialyzed against 2 L of 50 mM HEPES, 100 mM NaCl for 4 h for 2 rounds of dialysis. Sodium azide was added to a concentration of 0.02% to prevent bacterial growth. Purified proteins were stored at 4°C. Protein concentrations were determined using A280 spectroscopic measurements and calculated extinction coefficients.

Binding Biotinylated Substrates to Streptavidin Microspheres

SpheroTech streptavidin-coated pink particle kit microspheres were used with the following protease substrate GFP protein molecules. The P.01 microsphere was incubated with SNAP-25 ΔS1–S4. P.05 was incubated with the lethal factor consensus substrate to be

used as a negative control. P.07 was incubated with SNAP-25 ΔS4. P.09 was incubated with full-length SNAP-25 and P.11 was incubated with SNAP-25 ΔS1–S3. Fifty microliters of microspheres at a stock concentration of 10⁸ microspheres/mL was used in a final volume of 500 µL of protease buffer (50 mM HEPES, 100 mM NaCl, 1 mg/mL BSA, 0.025% Tween 20, pH 7.4) with 25–50 nM biotinylated GFP protease substrate molecule for 1 h at room temperature on a neutator mixing device covered from light. Microspheres and GFP substrates were centrifuged at 14,500 rpm in an Eppendorf mini-spin plus centrifuge with a F45–12–11 rotor for 2 min. The supernatant was removed by pipette while the microsphere pellets were left intact. Five hundred microliters of protease buffer was added to the microsphere pellets and the process was repeated 3 times. All microsphere pellets were suspended in 200 µL protease buffer and combined into one 1 mL fraction. Three milliliters of protease buffer was added to this 1 mL fraction for loading into 96-well screening plates.

For inhibition verification experiments, 50 µL of microspheres were used in the same 500 µL volumes with identical wash steps and brought up to final volumes of 1 mL with 100 µL of microspheres being used in 500 µL volumes run on a FACScan flow cytometer using 5 nM BoNTALC protease.

Plate Setup and Incubation

For screening of the Prestwick chemical library ISC BioSystems Genemate 96-well PCR plates were used. Fourteen microliters of the microsphere mix was added to each well of the 96-well plate by multichannel pipette. To column 1 of the plate, no test compound was added and an additional 1 µL of microsphere mix was added. Test compounds from the Prestwick library were diluted 1 to 5 in protease buffer and 1 µL of each dilution was added to a test well in the screening plate. The final concentration of test compounds in the screening plate was 20 µg/mL, or approximately 50–100 µM. Wells A12, B12, C12, and D12 had ZnCl₂ (a known inhibitor of metalloproteases) added to a final concentration of 1 mM. Final dimethyl sulfoxide (DMSO) concentrations in the assay were 1%. This level of DMSO was shown not to affect the protease assay as cleavage in endpoint assays was unchanged when they contained DMSO at concentrations as high as 10% (data not shown). Test compounds from the Prestwick library were added to wells prior to BoNTALC. Five microliters of 0.01 mg/mL BoNTALC suspended in protease buffer was added to each well except wells E12, F12, G12, and H12, giving a final concentration of 5 nM BoNTALC protease. Plates were incubated at room temperature for 1 h on a plate rotator to keep microspheres in suspension. One hour time points were chosen for screening in order to give maximum protease cleavage of substrate and the ability to screen plates sequentially. Substrate concentrations were determined based on the binding

capacity of the streptavidin-coated microspheres used in our assay. Plates were set up in duplicate, and each copy run 3 times on the Hypercyt® system.

High-Throughput Screening and Data Analysis

High-throughput screening of plates was done on the Hypercyt® high-throughput flow cytometry screening system connected to a Dako Cytomation CyAn ADP flow cytometer with settings of FS Peak Gain 15, SS Log voltage 300, gain 1.0, FITC Log voltage 700, gain 1.0, and PE Log voltage 700 gain 1. Wells were sampled for 1 s with ~2 µL volumes aspirated, with a 1 s wash step of protease buffer between each well. Each duplicate copy of each plate was sampled 3 times. Data analysis was done using the IDLe Query program developed by Dr. Bruce Edwards.¹⁸ Each of the microsphere sets were gated in the PE channel using a compensation value of PE fluorescence–7.6% FITC fluorescence. Median green fluorescence values for each microsphere set was calculated for each well of a 96-well plate. Data was exported to Excel in spreadsheet format for further analysis. *Z'* values were calculated for each bead set by using the average median and standard deviation values for wells containing no protease as a positive control and wells containing no test compound as a negative control. Percent inhibition was calculated using the formula $[100 \times (\text{averaged median well fluorescence} - \text{negative control average median fluorescence}) / (\text{positive control average median fluorescence} - \text{negative control average median fluorescence})]$. Percent inhibition values were averaged for each well with data for wells containing <50 events or *Z'* values for a particular substrate below 0.3 not used. The averaged values for each duplicate plate copy were then averaged to give an averaged percent inhibition value for each compound well. Compounds that gave an averaged percent inhibition value above 20% were selected for follow-up work.

Dose–Response Curves for Follow-Up Compounds

Follow-up work for compounds with >20% inhibition was done in 500 µL volumes of protease buffer and substrate bound microspheres. 5 nM of BoNTALC was used with increasing concentrations of test compounds and added after running the sample for 30 s on the flow cytometer. Samples ranged from 1 µM test compound to 50 µM test compound with samples of 1, 5, 10, 25, and 50 µM. A positive control sample with no protease was run along with a negative control with no inhibitory compound. Protease was added to each sample and incubated at room temperature while mixing on a neutator. Samples were run on a Becton Dickinson FACScan flow cytometer with 100 µL samples run on the cytometer every 30 min. FACScan PMT settings were FSC E00/9.25, SSC 225 log, FL1 700 log and FL2 600 log, compensation values of FL2–12% FL1, and gates were drawn on individual

bead set in the FL2 histogram plot. Percent inhibition was calculated by taking the 1 h sample value–1 h no inhibitor value and dividing by the 1 h no protease value–1 h no inhibitor value and multiplying by 100.

Inhibitor Testing Against LF Protease

To test the specificity of the identified inhibitor against BoNTALC, the LF15 GFP substrate protein was bound to Spherotech streptavidin pink particle P.07 and SNAP-25 GFP substrate protein was bound to microsphere P.11. Microspheres were bound and washed as described earlier. These were combined into 1 mL with 100 µL of microsphere mix being used in each sample. Sample volume of 600 µL samples of protease buffer with the desired amount of inhibitor added were used. One hundred microliters of sample, microspheres, and inhibitor were taken and run as a 0 min time point on a FACScan flow cytometer. 5 nM of BoNTALC and 500 nM of lethal factor protease were then added to tubes together to begin the assay. Samples were run on a Becton Dickinson FACScan flow cytometer as before but without compensation. Samples were run for time periods of 1 min on low flow rate every 30 min. Data were normalized by dividing each time point mean fluorescence values for each gated microsphere by the 0 min value collected for that microsphere before proteases were added.

Detailed Dose–Response Curve for Ebselen

To get a more detailed dose–response curve for the inhibitor ebselen against full-length SNAP-25 GFP, 50 µL of each microsphere set was used with SNAP-25 GFP bound to microsphere P.11 and LF15 GFP bound to microsphere P.07 as described earlier. Microspheres were washed 3 times, combined, and brought up to a 1,400 µL volume. Hundred microliters of the microsphere mix was used in each sample in 500 µL total volumes containing amounts of ebselen in the increments of 1, 2.5, 5, 6, 7, 8, 9, 10, 12.5, 15, 25, and 50 µM. Samples were run for 30 s on a FACScan flow cytometer with settings described earlier. 5 nM of BoNTALC was added after 30 s and data analyzed for 15 min on continuous run. Percent inhibition was calculated by using the first 60 s slopes and the equation $(\text{slope no inhibitor} - \text{slope sample}) / (\text{slope no inhibitor}) \times 100$.

Inhibitor Measurements by FRET

The SNAPtide peptide from List Biological Laboratories was used at a concentration of 5 µM in protease buffer with excitation at 490 nm and emission measured at 523 nm on a Photon Technology International (PTI) fluorimeter model # LPS 220B, with the PTI monochromator slit size of 2 nm and a stir bar continuously stirring the sample. 5 nM of BoNTALC was added at 60 s of measurement and run for 10 min with samples containing increasing

concentrations of identified inhibitor present. The 10 min slopes of each sample were taken and converted to percent inhibition using the equation (slope no inhibitor – slope sample)/slope no inhibitor $\times$ 100.

RESULTS

A Multiplex Microsphere-Based Flow Cytometry Assay for BoNTALC Activity

Four fusion proteins from SNAP-25 (bearing N-terminal biotinylation tags and C-terminal GFP labels) were cloned, purified, and attached to a multiplex set of microspheres (Fig. 1). A substrate for *Bacillus anthracis* lethal factor protease was used as a negative control (Fig. 1). BoNTALC assays were run continuously on a flow cytometer and bead sets gated based on size and yellow fluorescence intensity (580 nm/30 nm) to discriminate microspheres bearing each substrate (Fig. 2A). The assay was initiated by the addition of protease and the microsphere bearing full-length SNAP-25 lost fluorescence due to proteolytic cleavage at the fastest rate, followed closely by SNAP-25 Δ S1–S3 (Fig. 2B and 2C). SNAP-25 Δ S4 and Δ S1–S4 showed far less cleavage in the same time frame, with SNAP-25 Δ S4 showing slightly faster fluorescence loss than SNAP-25 Δ S1–S4. (Fig. 2B and 2C) The control LF substrate showed very little fluorescence loss, indicating that the loss on the SNAP-25 substrates is due to specific proteolytic cleavage of the GFP-linked substrate (Fig. 2B and 2C). The rate of specific cleavage increased as a function of BoNTALC concentration (1–50 nM), but the relative substrate cleavage profiles remained the same (data not shown). A similar pattern of substrate susceptibility to cleavage was observed in endpoint assays used for compound screening (see below). After an 1 h incubation with BoNTALC at room temperature, full-length SNAP-25 and SNAP-25 Δ S1–S3 were both cleaved to lower fluorescence values than SNAP-25 Δ S4 and Δ S1–S4 (data not shown).

High-Throughput Screening of the Prestwick Chemical Library

One hour endpoint assays were used for library screening, where BoNTALC was added to 20 μ L multiplex microsphere assays, with test compounds, in 96-well plates and incubated at 25°C. Under these conditions, fluorescence loss due to proteolytic cleavage was seen across all 4 forms of SNAP-25, with ~5% fluorescence loss on the control substrate due to GFP instability or photobleaching (data not shown). Plates were analyzed via the HyperCyt HTS system attached to a Cyan ADP flow cytometer. Z' values were calculated separately for each substrate on every run of individual plates. Data for copies of plates with Z' values below 0.3 were not used to calculate percent inhibition averages or Z' averages. Out of 66 plate runs, 7 plate runs were not used due to extremely low or negative Z' values, which were most likely

due to instrumental error such as clogs or very low microsphere pickup. For the remaining 59 plate runs, averaged Z' values were calculated along with standard deviations for each substrate used in the screening assay. Full-length SNAP-25 gave an averaged Z' of 0.713 with a standard deviation of 0.264. SNAP-25 Δ S1–S3 gave an averaged Z' of 0.890 and a standard deviation of 0.096. SNAP-25 Δ S4 gave an averaged Z' of 0.798 and a standard deviation of 0.142. SNAP-25 Δ S1–S4 gave an averaged Z' value of 0.821 and a standard deviation of 0.136. Representative raw GFP

Fig. 1. Schematic representation of the 5-plex substrate system. Microsphere populations are represented by different patterns: streptavidin by the crescent, biotinylation tag by the oval, green fluorescent protein (GFP) by the sun, cleavage site on synaptosome-associated protein of 25 kDa (SNAP-25) by the grey square, and distal binding sites by the black gaps. (A) Full-length SNAP-25. (B) SNAP-25 Δ S1–S4 (residues 21–59 and 145–155 deleted) containing no distal binding sites (C) SNAP-25 Δ S4 (residues 145–155 deleted) containing distal binding sites S1–S3. (D) SNAP-25 Δ S1–S3 (residues 21–59 deleted) containing distal binding site S4. (E) Lethal factor cleavage site control substrate was used as a negative control.

fluorescence data derived from gating of the microspheres for the population bearing the Δ S1-S3 show the results of endpoint assays distributed serially over time (Fig. 3). As protease activity

results in loss of fluorescence, the wells that contained negative controls or had non-inhibitory compounds resulted in a ~10-fold reduction of fluorescence as compared to positive control wells that were inhibited by an addition 1 mM Zn^{2+} or lacked protease altogether (Fig. 3). While in this screen we did not see any experimental wells that inhibited at the level of the positive controls (100%), several wells resulted in partial inhibition, which indicated a potential inhibitor (Fig. 3). Analysis of all of the data showed that 3 compounds inhibited all 4 substrates: methylene blue (21% to 42% inhibition), coralyne chloride hydrate (28% to 56% inhibition), and ebselen (88% to 90% inhibition).

Ebselen Inhibits Light Chain Across all 4 Substrates Screened

The 3 positive hit compounds from the screen were used in follow-up flow cytometry assays to determine concentration-response relationships. Methylene blue and coralyne chloride hydrate gave ~40% inhibition at concentrations of 65 and 45 μ M, respectively (data not shown). Due to poor inhibition at high concentrations, these compounds were not pursued further. However, increasing concentrations of ebselen in the assay resulted in an estimated IC_{50} value of 4.9 μ M against full-length SNAP-25 with a Hill-Slope of 25 ± 6 (Fig. 4).

Ebselen Does Not Inhibit the Metalloprotease *B. anthracis* Lethal Factor

To test if ebselen inhibition is specific to BoNTALC, we used a duplex microsphere assay with the substrate for *B. anthracis* lethal factor protease (the negative control substrate in the BoNTALC assay) attached to one microsphere and full-length SNAP-25 GFP

Fig. 2. Five-plex flow cytometry assay with botulinum neurotoxin type A light chain (BoNTALC) run continuously on a FACScan flow cytometer. (A) Multiplex bead set of FL2 vs. number of events showing the 5 distinct populations of microspheres. (B) Continuously run data plotted for all 5 microsphere types gated on each microsphere/substrate type in real time with 1% of total events shown. Substrates represented: full-length synaptosome-associated protein of 25 kDa (SNAP-25) green fluorescent protein (GFP); SNAP-25 Δ S1-S3; SNAP-25 Δ S4; SNAP-25 Δ S1-S4; and LF control substrate. Data were analyzed in FloJo flow cytometry data analysis software. (C) Relative rates of 5-plex substrate cleavage after addition of 25 nM BoNTALC. Data were normalized by averaging mean FL1 fluorescence values for the first 55 s of data collection on a FACScan flow cytometer prior to addition of 25 nM BoNTALC and dividing all subsequent mean FL1 fluorescence values by that averaged value. Substrate types are as follows: full-length SNAP-25; SNAP-25 Δ S1-S4; SNAP-25 Δ S4; LF control substrate; and SNAP-25 Δ S1-S3.

Fig. 3. Raw data from a Cyan ADP flow cytometer of a 96-well plate for the substrate synaptosome-associated protein of 25 kDa (SNAP-25) $\Delta S1-S3$. Darker clusters of events are from each well. Wells with high green fluorescence (FL1) values are negative control wells at 12-well intervals as defined in Materials and Methods.

attached to the other microsphere. This duplex assay was performed using assay conditions shown to be effective for LF protease activity in a microsphere-based protease assay.¹⁸ The assay was initiated via the addition of both LF and BoNTALC, except in the case of controls. Addition of increasing concentrations of ebselen

Fig. 4. Dose-response curve for the compound ebselen tested against 5 nM botulinum neurotoxin type A light chain (BoNTALC) in our microsphere-based assay using biotinylated full-length synaptosome-associated protein of 25 kDa (SNAP-25) green fluorescent protein (GFP). These data were fitted to a 3-parameter fixed bottom logistic model for a Hill-Slope dose-response curve, which was defined as $\% \text{ inhibition} = \text{bottom} + [(\text{top} - \text{bottom}) / (1 + (x / \log_{10}(IC_{50}))^{\text{Hill-Slope}})]$, where the bottom was fixed to the value of the negative control, as the dosing did not extend into the low concentrations, which required the fixing of the bottom parameter to obtain an accurate fit. This fit resulted in an estimate of the $\log_{10}(IC_{50})$ to be -5.31 ± 0.05 , which estimates the IC_{50} to be 4.9 μM . The Hill-Slope was estimated to be 25 ± 6 and the fit gave an R^2 of 0.905.

(1–1,000 μM) demonstrated that concentrations as high as 250 μM did not inhibit LF and 500 μM ebselen only resulted in ~30% inhibition after 1 h of incubation (Fig. 5). This is in contrast to a known LF inhibitor, which gave ~90% inhibition of LF at 1 μM after 1 h (Fig. 5). As seen previously, ebselen inhibited the BoNTALC at concentrations between 5 and 10 μM (data not shown).

Ebselen Inhibits BoNTALC in a Peptide-Based FRET Assay

To verify that ebselen is inhibiting the BoNTALC protease and not via some artifact in the SNAP-25 microsphere assay, we used

Fig. 5. Ebselen tested against the *Bacillus anthracis* lethal factor metalloprotease in a flow cytometry microsphere-based loss of green fluorescence assay. Lethal factor of 500 nM was added to microspheres containing a biotinylated green fluorescent protein (GFP)-based lethal factor consensus cleavage site sequence and measured on a FACScan flow cytometer every 30 min. Samples are represented by the following symbols. Closed circles: no lethal factor; open circles: no ebselen; inverted closed triangles: 5 μM ebselen; open triangles: 25 μM ebselen; closed squares: 250 μM ebselen; open squares: 500 μM ebselen; and diamonds: 1 μM IN-2-LF, a known lethal factor inhibitor.

the FRET peptide substrate for BoNTALC (SNAPTide) from List Laboratories (Campbell, CA). In the presence of 5 nM of BoNTALC, ebselen resulted in an IC_{50} of 18.6 μ M with a Hill-Slope of 27 ± 7 (Fig. 6).

Inhibition by Ebselen Is Not Due to Cysteine Modification

Ebselen has been reported to be a potent inhibitor of cysteine proteases via cysteine modification that is reversible via the use of reducing agents.²² To explore if inhibition of BoNTALC is a result of cysteine modification by ebselen either on SNAP-25 or BoNTALC, we specifically attempted to reverse the inhibition of the SNAPTide assay. The SNAPTide FRET peptide does not contain cysteines. This suggests that if cysteine modification was the source of inhibition, a cysteine residue of BoNTALC must be involved. Reversal of inhibition was attempted by addition of 1 mM DTT or 0.05% β -mercaptoethanol to the SNAPTide assay. Neither affected inhibition levels (data not shown) suggesting

that inhibition of BoNTALC by ebselen was not due to cysteine modification.

DISCUSSION

We have developed a multiplex microsphere protease activity assay that enabled screening BoNTALC against several SNARE motif deletion forms of SNAP-25 in parallel, and resulted in identification of a novel inhibitor. The assay enabled use of full-length substrates, a feature that may help to identify small molecule compounds targeting interactions with SNAP-25 that are distant from the cleavage site. The potential for discovery of small molecule inhibitors that interfere with protein–protein interactions between SNAP-25 and BoNTALC has been noted,⁷ and screening efforts of libraries of compounds known to interfere with such protein–protein interactions have resulted in discovery of BoNTALC inhibitors.²³ Such interactions, however, have not been verified and are still hypothetical.²³ While our current screen has not definitively detected such inhibitors, our 5-plex assay to measure cleavage across many structural variants gave results consistent with previous studies that showed a slower rate of SNAP-25 cleavage of substrates lacking the S4 SNARE motif (Figs. 2 and 3). This suggests that our assay may be a viable approach for detecting such inhibitors in extended screening of larger compound collections.

The screen of the Prestwick Chemical Library resulted in the detection of ebselen, which inhibited BoNTALC across all 4 forms of SNAP-25 used. The averaged Z' values, reported for each substrate, were all acceptable for HTS. Ebselen inhibited 5 nM BoNTALC with an IC_{50} of 4.9 μ M for full-length SNAP-25 and 18.6 μ M for a peptide-based substrate. Because ebselen exhibited similar BoNTALC inhibition on all 4 SNAP-25 substrates, it seems unlikely to be targeting interactions between SNAP-25 and BoNTALC within the SNARE motifs. Such inhibition would have likely been revealed by differences in the percent inhibition between substrates bearing different SNARE motifs. However, as ebselen inhibition of LF was not observed at ebselen concentrations 100-fold higher than required for BoNTALC inhibition (Fig. 5), we postulate that ebselen is acting upon BoNTALC specifically and is not a general Zn^{2+} metalloprotease inhibitor.

While ebselen specifically inhibited BoNTALC at low μ M concentrations, several aspects of the inhibition were of concern. First, the steep Hill-Slopes obtained from the dose–response curves indicate that inhibition may not be occurring through a simple mechanism. One potential concern was that ebselen was causing a solution effect that resulted in loss of BoNTALC activity. However, LF protease activity is unaffected by ebselen (Fig. 5), making it unlikely that a solution effect is causing the loss of BoNTALC activity, as LF and BoNTALC require nearly

Fig. 6. Inhibition of 5 nM botulinum neurotoxin type A light chain (BoNTALC) by ebselen tested with the synaptosome-associated protein (SNAP) SNAPTide peptide substrate. Ebselen and 5 μ M SNAPTide were added and measured by excitation at 490 nm and emission values were recorded at 523 nm in a fluorimeter. 5 nM of BoNTALC was added after 60 s and data collected for 10 min after protease addition. These data were fitted to a 4-parameter logistic model for a Hill-Slope dose–response curve, which was defined as $\% \text{ inhibition} = \text{bottom} + [(\text{top} - \text{bottom}) / (1 + (x / \log_{10}(IC_{50}))^{\text{Hill-Slope}})]$. This fit resulted in an estimate of the $\log_{10}(IC_{50})$ to be -4.73 ± 0.05 , which estimates the IC_{50} to be 18.6 μ M. The Hill-Slope was very abrupt and estimated to be 27 ± 7 and the fit gave an R^2 of 0.982.

identical buffers for activity. Second, excess Zn^{2+} is expected to be a general metalloprotease inhibitor.²⁴ Potential concerns about Zn^{2+} contamination causing the inhibitory action of ebselen on BoNTALC were also addressed by the duplex BoNTALC-LF assay, where both proteases were used simultaneously. If Zn^{2+} contamination were responsible for inhibition, it would have likely been seen as an inhibitor of LF in this assay, as the same amount of potential Zn^{2+} contaminant would be present for both proteases. However, in this experiment BoNTALC is inhibited while LF is not at high concentrations of ebselen (Fig. 5), which suggests Zn^{2+} is not present at inhibitory concentrations. Third, ebselen has been shown to be a somewhat promiscuous inhibitor, and has been identified as a lead compound in numerous assays. Ebselen has been shown to be an antioxidant and peroxynitrite scavenger²⁴ that mimics glutathione peroxidase.²⁵ Ebselen has also been reported to have neuroprotective activity after embolic stroke,²⁶ to attenuate intestinal ischemia/reperfusion injuries,²⁷ to act as antifungal agent even on fluconazole-resistant yeast cells,²⁸ and to act as an antibacterial and antiviral agent,²⁹ among other activities. Ebselen is currently under FDA-approved clinical trials by Sound Pharmaceuticals (as SPI-1005 and SPI-2005) for hearing loss treatment (<http://www.soundpharmaceuticals.com/>). A recent PubChem search reveals that ebselen was found bioactive in 70 assays out of 438, of which 39 were confirmatory assays. One mechanism of inhibition by ebselen has been demonstrated through cysteine modification of papain (a cysteine protease) and other proteins, which is reversible by reducing agents.^{22,30} This known ability of ebselen to modify cysteine residues may be partly responsible for its promiscuity in screening assays. However, the inability to reverse inhibition via reducing agents suggests that ebselen is not inhibiting BoNTALC via oxidation of cysteines. Thus, while there are many interesting aspects to the ebselen inhibition of BoNTALC discovered here, it appears that the inhibition is BoNTALC-specific, not based on oxidation of cysteines, and likely not a result of Zn^{2+} contamination of the ebselen, all of which makes it a potentially interesting BoNTALC inhibitor.

Ebselen is of specific interest as a BoNTALC inhibitor because it is a bioavailable compound pre-approved for human use for a variety of purposes. Importantly, the IC_{50} (4.9 μM) of ebselen toward BoNTALC is very good in comparison to other previously reported BoNTALC small molecule inhibitors.^{31,32} This compound has also been shown to have low toxicity in animal studies, and has been administered at levels up to 10 mg/kg in cynomolgus monkeys.³³ Because of its combination of bioavailability and acceptable toxicity level, ebselen may be a useful pharmaceutical for BoNTALC poisoning treatment. Studies are being pursued to investigate its effectiveness in animal models.

This particular assay format also has the distinct advantage of being adaptable to any protease of interest for HTS purposes. Full-length protease substrates can be used to identify small molecules that may interfere with protease substrate interactions leading to specific inhibition of the protease of interest. This assay can also be used for parallel evaluation of multiple proteases, as demonstrated with the dual lethal factor/BoNTALC assay in this study (Fig. 5). Screening of multiple proteases against multiple substrates could also lead to discovery of specific inhibitors of closely related proteases and reduce costs of materials and labor in HTS.

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AUTHOR DISCLOSURE STATEMENT

None of the authors of this work have any conflicting interests in this work.

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