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Superactivation of the Botulinum Neurotoxin Serotype A Light Chain Metalloprotease: A New Wrinkle in Botulinum Neurotoxin

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The seven neurotoxins of the anaerobic spore-forming bacterium *Clostridium botulinum* (BoNTs A−G) are the most lethal poisons known, with BoNT serotype A having a LD_{50} for a 70 kg human of a mere 0.8 μg by inhalation.[1] Historically associated with food poisoning, these proteinaceous toxins inhibit the release of acetylcholine at neuromuscular junctions by cleavage of SNARE proteins, resulting in progressive flaccid paralysis.[2] It is as a result of this potent neurotoxic activity that BoNTs are of substantial concern as potential bioterrorist weapons.[3] Paradoxically, the use of BoNT in medicine has become increasingly popular in recent years, with the most well-known application being its use in the cosmetic treatment of glabellar lines (i.e., forehead wrinkles).[4] Less well publicized is the emergence of BoNT as an excellent therapeutic tool in the treatment of a variety of serious medical conditions.[5] Multiple sclerosis, stroke, cerebral palsy, migraine, and backache are just a few of the conditions for which BoNT therapy has proven effective. However, repeated toxin exposure can lead to the development of a significant immune response, resulting in a considerable drawback associated with BoNT therapy.[6] Tolerance develops most rapidly when patients are treated frequently with high doses of the toxin. We hypothesized that administration of BoNT in combination with a molecule which can "activate" the proteolytic activity of the toxin would allow the use of lower doses, thus reducing the unintended adaptive immune response. Herein, we disclose the discovery of the first small molecule activators of BoNT A catalytic activity based upon a 2-acylguanidine-5-phenyl thiophene scaffold that display structure−activity relationships (SAR) indicative of discrete toxin binding and result in the greatest protease activation reported to date.

During the course of our recent investigations into the identification of BoNT A light chain metalloprotease (BoNT LC/A) inhibitors, we found that modest inhibition could be obtained with molecules as simple as arginine hydroxamic acid;[7] in essence, this compound is simply comprised of a zinc-binding moiety and a guanidinyl group. Using this as a guide, we prepared a series of compounds containing a 2-acylthiophene moiety as a zinc-binding functionality, combined with acylguanidyl groups as an arginine side chain mimetic. While under our standard FRET-based screening conditions[7] no inhibition was observed, however, we were surprised to discover that one compound (**1a**) consistently produced a 2-fold enhancement of BoNT LC/A catalytic activity (Table 1). Gratifyingly, comparable activation was also observed with the native substrate SNAP-25(141−206),[7] indicating that the activation was not the result of a fluorescence artifact.

Using activator **1a** as a lead, we explored the molecular requirements for activation by synthesizing and testing compounds **1**−**6** (Figure 1) for their effect on BoNT LC/A activity. Interestingly, the corresponding furan analogue **2**, carboxylic acid **5**, and methyl ether substituted derivative **1h** were nonactivating. In addition, no activation was observed using phenyl or 2-pyridyl

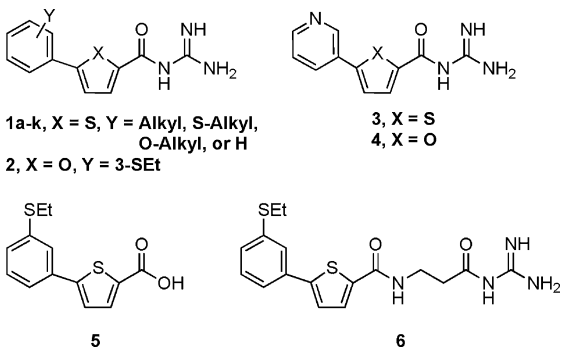

Figure 1. 2-Acylguanidyl-5-phenyl thiophenes synthesized to examine BoNT LC/A activation.

Table 1. Structure−Activity Relationship of 5-Phenyl-2-acylguanidyl Thiophene Activators of BoNT LC/A under Standard Screening Conditions

compound	X	Y	max activation (compound concn)
1a	S	3-SEt	4-fold (500 μM)
1b	S	4-SEt	3.5-fold (100 μM)
1c	S	4-^{n}Pr	7-fold (200 μM)
1d	S	3-SMe	3-fold (500 μM)
1e	S	4-S^{n}Bu	7-fold (20 μM)
1f	S	3-S^{n}Bu	4-fold (20 μM)
1g	S	3-S^{n}Bn	inactive
1h	S	3-OMe	inactive
1i	S	2-SMe	inactive
1j	S	2-SEt	inactive
1k	S	H	inactive
2	O	3-SEt	inactive
3	S		inactive
4	O		inactive
5	S	3-SEt	inactive
6	S	3-SEt	inactive

derivatives **1k** or **3**. Substitution of the phenyl ring at the 2-position also yielded inactive compounds (**1i** and **1j**); however, 4-thioethyl substituted analogue **1b** was found to be a more potent activator than lead compound **1a**. On the basis of these data, we focused our attention on the nature of substituents at the 3- and 4-positions. 4-Butylthioether **1e** and 3-butylthioether **1f** were both significantly better activators than **1a** or **1b**, illustrating that increasing the length of the aliphatic chain at the 3- and 4-positions enhances activity. However, replacement of this aliphatic chain with an aromatic substituent as in **1g** abrogated activation. Compound **6** was examined to ascertain the importance of the distance between the guanidyl group and thiophene core; however, this modification resulted in a complete loss of activity.

Given the tremendous clinical promise of a BoNT activator coupled with the lack of a fully characterized allosteric site on the BoNT light chain,[8] we examined BoNT activation by these

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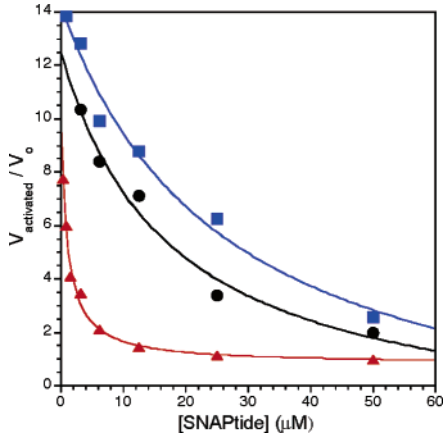

Figure 2. BoNT LC/A activation in the presence of 100 μM **1b** (red triangles), 100 μM **1c** (black circles), and 40 μM **1e** (blue squares) as a function of substrate concentration. Data represent the average of duplicate measurements.

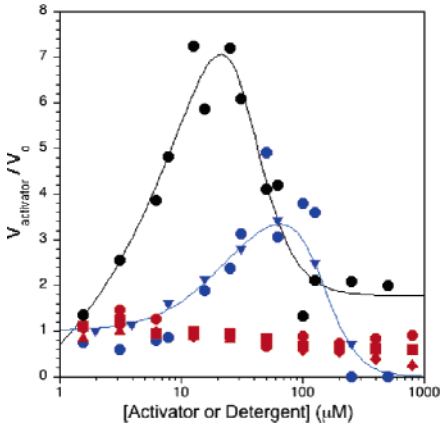

Figure 3. BoNT LC/A activation profiles as a function of **1e** and several detergents: **1e** (black circles), CTAB (blue circles), CPB (blue triangles), Tween-20 (red circles), NLS (red triangles), CHAPS (red squares), and deoxycholate (red diamonds). Data represent the average of duplicate runs.

compounds in greater detail in order to understand the mechanism of this phenomenon. Kinetically, the catalytic activation provided by compounds **1a**−**1f** could result from modulation of k_{cat}, K_m, or both. Enhancement in k_{cat} only will produce equal activation at all substrate concentrations, while enhancements in K_m will produce greater activation at low substrate concentration and no activation at saturating substrate concentration. Enhancements in both k_{cat} and K_m will produce the greatest activation at limiting substrate concentration while asymptotically approaching a constant amount of activation at saturating substrate concentration. Thus, varying the substrate concentration at a fixed compound concentration allows one to distinguish which mechanism is in effect. Examination of the three most active compounds (**1a**, **1c**, and **1e**) revealed a reduction in K_m and little to no effect on k_{cat} (Figure 2). Critically, the 14-fold rate enhancement shown by **1e** at limiting substrate concentrations is the greatest activation reported for a protease. Indeed, 2-fold activation of a protease has been previously referred to as a state of "superactivation".[9]

Two of the elements required for activation, the aliphatic substituents on the phenyl ring and the positively charged 2-acyl guanidyl group, suggest the structural motif of a detergent and, therefore, rate enhancement from a nonspecific effect. To address this issue, the impact on BoNT LC/A activity of six detergents with varying electrostatic profiles was investigated. All of the detergents tested were inhibitory above their critical micelle concentration (CMC); however, at lower concentrations, varying activation profiles became apparent (Figure 3). Neutral and zwitterionic detergents, such as Tween-20 and CHAPS, respectively, had little effect on enzyme activity, while the anionic detergents deoxycholate and *N*-lauryl sarcosine (NLS) were particularly inhibitory. Alternatively, the cationic cetyl-based detergents cetyltrimethylammonium bromide (CTAB) and cetyl pyridium bromide (CPB) were activating when present 5-fold below their 500 μM CMC, but became strongly inhibitory as CMC was approached.

Activation of proteases by cationic detergents has been observed and studied extensively with α-chymotrypsin.[10] In contrast to our studies, activation of α-chymotrypsin occurs only at detergent concentrations above CMC and, therefore, is an interfacial mechanism of activation whereby enzyme and substrate interact at the interface between detergent micelles and surrounding solvent. Conversely, due to the observed enhancement below CMC, the activation of BoNT LC/A by CTAB and CPB is likely due to discrete binding of one or more detergent molecules to the enzyme. Precedence for this sort of noninterfacial activation mechanism has been demonstrated recently in a thorough study of the activation and inhibition of *T. lanuginosus* lipase by detergents.[11] Here, no evidence of micelles or premicellar aggregates was found as judged from dynamic light scattering and fluorescence experiments. Given that the most highly activating compounds **1e** and **1f** show maximal activity at lower concentrations than these cationic detergents, the compilation of this with our previous SAR data suggests that these compounds activate BoNT LC/A by discrete binding to the toxin or its substrate, thereby facilitating catalytic activity.

In summary, we have unveiled a small molecule scaffold based on 2-acyl guanidyl-5-phenyl thiophenes that strongly activates BoNT LC/A catalytic activity through an apparent reduction in K_m. The activation profile and structure−activity relationship for activation suggests the presence of a specific binding domain on the enzyme and not a "detergent-like" mechanism. As the importance of BoNT in medicine continues to expand, adaptive immune responses to the toxin must be addressed. The discovery and optimization of small molecule activators may ultimately provide a valuable method for minimizing BoNT dosage, thereby increasing BoNT clinical efficacy.

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Supporting Information Available: Full experimental procedures and characterization for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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