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A Platform Stratifying a Sequestering Agent and a Pharmacological Antagonist as a Means to Negate Botulinum Neurotoxicity

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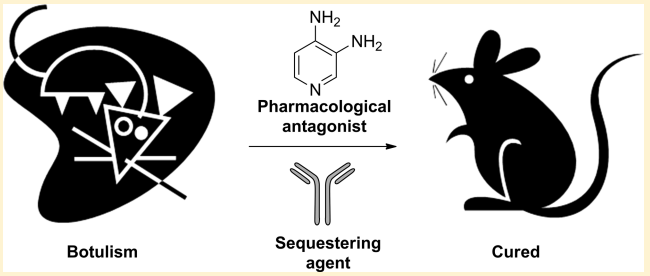
Supporting Information

ABSTRACT: Botulinum neurotoxicity is characterized by peripheral neuromuscular blockade/flaccid paralysis that can lead to respiratory failure and ultimately death. Current therapeutic options provide relief in a pre-exposure scenario, but there are no clinically approved postexposure medical countermeasures. Here, we introduce a platform that utilizes a combination of a toxin sequestering agent and a pharmacological antagonist to ablate botulinum neurotoxicity in a well-defined mouse lethality assay. The platform was constructed to allow for ready exchange of sequestering agent and/or pharmacological antagonist for therapeutic optimization. As such, we attempted to improve upon the pharmacological antagonist, a potassium channel blocker, 3,4-diaminopyridine, through a prodrug approach; thus, a complete kinetic decomposition pathway is described. These experiments provide the first proof-of-principle that a synergistic combination strategy can be used to reduce toxin burden in the peripheral using a sequestering antibody, while restoring muscle action via a pharmacological small molecule antagonist.



KEYWORDS: *Botulinum neurotoxin, prodrug, antibody, K^+ channel blocker, drug combination*

The bacterium *Clostridium botulinum* produces the deadliest neurotoxins known to mankind.[1] There are seven botulinum neurotoxins (designated as serotypes A−G) produced from this bacterium of which botulinum neurotoxins (BoNTs) A/B/E are responsible for human botulism. This disease is characterized by muscle paralysis and, if left untreated, results in patient death due to impairment of respiratory functions. Today, food poisoning cases related to botulism are rare, but there are growing concerns centered upon the exploitation of BoNT/A as a bioterrorist weapon.[2] Due to the extreme potency of BoNT/A (lethal dose =1 ng/kg body weight), coupled with its ease of production and dissemination, the United States Centers for Disease Control and Prevention have classified BoNT as a category A bioterrorism agent.[3]

The active form of the BoNTs comprises two protein domains: a 100 kDa heavy-chain (HC) linked to a 50 kDa light-chain (LC) metalloprotease via a disulfide bond.[4] BoNT/A intoxication is initiated through the binding of the HC domain to cellular receptor proteins and internalization of the protein by receptor mediated endocytosis. Following internalization, the disulfide bond is cleaved and the LC undergoes translocation in to the cytosol. The LC metalloprotease then cleaves one of three SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which ultimately results in the inhibition of acetylcholine release at the neuromuscular junction and termination of neurotransmission.[5]

Currently, there are several approaches that are being pursued for the prevention/treatment of botulism; among the most important are vaccines, therapeutic antibodies (Abs), and pharmacological antagonists.[6] Vaccines have proven to be an effective means of preventing botulinum intoxication; however, patients are generally restricted to consenting military personnel only.[5] Thus, in the event of a bioterrorist attack, the general public would not be vaccinated and would be unprepared for such an incident. Simply stated, vaccines are insufficient in a postexposure incident. In such a scenario, therapeutic Abs have shown the most promise as they have been approved for the treatment of BoNT intoxication in humans.[7]

Antibodies are successful in diminishing the concentration of toxin in circulation. This is accomplished through the formation of an antibody−antigen union that is then recognizable for elimination from the blood. As the toxin is removed from circulation, it is no longer able to enter the nerve cells and thus halts any further paralytic effects from BoNT intoxication.[8] While this is a promising strategy for the treatment of botulism, it is not without its shortcomings. Therapeutic antibodies are incapable of entering nerve cells and thus unable to reverse paralysis once cellular uptake has occurred. This demonstrates a clear temporal limitation of antibody administration for the treatment of BoNT intoxication.

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Conversely, a pharmacological antagonist can enter the nerve cell and in theory should temporarily reduce the paralytic effects of BoNT. Unfortunately, no small molecule has been approved for the treatment of botulism, although K^+ channel blockers and protease inhibitors have been touted as potential leads.[9−12] A major impediment in the advancement of these compounds is their inherently short half-life relative to that of BoNT, which depending on the serotype can exist for months in humans.[13] Thus, small molecules may provide temporary relief but sustained administration would be required over the duration of BoNT intoxication, as the compounds are unable to eliminate toxin circulation in the periphery.

A combination strategy can be envisioned to address the limitations of therapeutic antibody/small molecule antagonists, and provide a treatment option for a BoNT postexposure occurrence. Engaging this strategy implies that the strength of each therapeutic method will complement the very weakness of the other. Thus, using a sequestering agent would reduce toxin load in circulation and negate the toxin's long half-life, while concurrent administration of a pharmacological antagonist would counter the toxin's action inside nerve cells. As such, this synergistic combination strategy would address both short- and long-term effects of BoNT intoxication. Finally, if this platform can be convincingly established, then it could potentially apply to any therapeutic antibody and any pharmacological antagonist(s) used in combination.

RESULTS AND DISCUSSION

To test our platform strategy, we used the mouse lethality assay, currently the FDA gold standard for assessing BoNT activity. As a pharmacological antagonist, we used the K^+ channel blocker 3,4-diaminopyridine (3,4-DAP), as it has been shown to be effective in antagonizing muscle paralysis as a result of BoNT/A exposure.[14] This compound is known to offset the effects of BoNT by blocking presynaptic K^+ channels and thereby increasing Ca^{2+} influx, resulting in enhanced release of acetylcholine within the synaptic cleft. Studies have shown that this compound will produce the desired effect of restoring muscle action potential in vitro and in mouse models.[10,14,15]

For this combination experiment, there were four test groups. Mice were either injected intraperitoneally with BoNT/A alone (at 5 LD_{50} per mouse, $n = 6$), BoNT/A and Ab ($n = 4$), BoNT/A and 3,4-DAP ($n = 6$), or BoNT/A and a combination of Ab and 3,4-DAP ($n = 6$). The antibody used in this experiment was of polyclonal makeup and has the capacity to neutralize 10 000 LD_{50}, if administered 30 min pretoxin. As a frame of reference, the polyclonal antibody, if administered 15, 30, or 60 min after toxin challenge (5 LD_{50}), grants complete survival (data not shown). However, if the antibody is administered 2 h post toxin challenge (5 LD_{50}), protection is not observed, and hence, sufficient toxin has entered cells. The aminopyridine was administered 3 h after toxin administration, thus 1 h after antibody injection. It should be noted that all mice had tense abdomens after 2 h post BoNT/A administration and labored breathing at 3 h. Experimental data was fit to a survival curve as shown in Figure 1.

All mice administered with BoNT/A or BoNT/A + Ab were deceased by 492 ± 82 min. The survival rate for the mice injected with toxin/3,4-DAP was 17%; however, when mice were dispensed with the combination cocktail (Ab + 3,4-DAP), the survival percentage was augmented to 50%. These results demonstrate that the administration of an antibody or 3,4-DAP

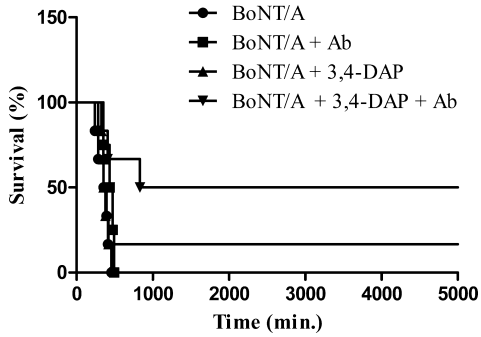

Figure 1. Survival graph for four test groups following BoNT administration. Survival rate was not significantly higher for mice injected with BoNT/A + Ab compared to BoNT/A alone ($\rho = 0.1$; log-rank test). Survival rate was not significantly higher for mice injected with BoNT/A + 3,4-DAP compared to BoNT/A alone ($\rho > 0.1$; log-rank test). Survival rate was significantly higher for mice injected with BoNT/A + 3,4-DAP + Ab than for mice injected with BoNT/A alone ($\rho < 0.05$; log-rank test).

alone does not significantly increase the BoNT/A survival rate (Figure 1; $\rho \geq 0.1$; log-rank test). However, mice injected with a combination of antibody and 3,4-DAP were significantly more likely to survive the effects of BoNT/A intoxication ($\rho < 0.05$; log-rank test). This then represents the first reported successful combination treatment for BoNT/A intoxication using a sequestering antibody to reduce toxin load, while simultaneously reversing BoNT/A induced paralysis via a small molecule.

As stated, vide supra, an important value of our platform is that it should be valid with any BoNT sequestering agent or pharmacologic antagonist. Because the countermeasure used in our strategy can be readily exchanged as a means to improve efficacy, we next sought to address some of the limitations associated with aminopyridines. For example, they typically have a short plasma half-life (0.5−1 h) and can be prone to blood-brain barrier penetration, which can lead to seizures.[10] Furthermore, providing more than transient efficacy requires the use of an osmotic minipump.[11,16] As such, any potential clinical treatment utilizing an aminopyridine would require continuous intravenous infusion or a repeated dose regimen. Notwithstanding the negative side effects associated with aminopyridines, our data indicates its potential as an effective treatment of BoNT/A in mouse models, especially over other K^+ channel blockers such as scorpion venom derived peptides.[12] Thus, we invoked the development of a prodrug in an effort to improve the pharmacokinetic properties of 3,4-DAP. Recently, our laboratory applied a prodrug approach to create carbamate/amide-ester conjugates of 3,4-DAP with the dual intent of inhibiting acetylcholinesterease and releasing localized 3,4-DAP at the synaptic cleft.[17] To further advance this line of thought, we looked to an amino acid prodrug strategy as a means to both extend the aminopyridine's half-life in serum and also increase its polar surface area, thus negating CNS penetration.[18]

As a starting point, we looked to test the simplest member, a 3,4-DAP alanine prodrug, which we anticipated would release 3,4-DAP via esterase or peptidase cleavage to release benign, amino acid chemical entities as byproducts.[19] A mixture of the bis-substituted 3,4-DAP and the monosubstituted regioisomers was obtained using standard EDC coupling conditions between Boc-L-alanine and 3,4-DAP. Following purification and Boc-

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deprotection, the 3,4-DAP prodrug was obtained (Figure 2). Next, we examined the unmasking pathway of the prodrug with

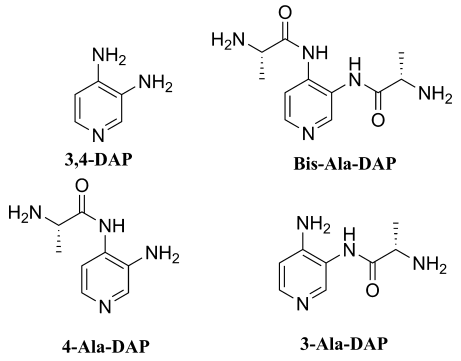

Figure 2. Chemical structure of 3,4-DAP (K^+ channel blocker), Bis-Ala-DAP (prodrug), 4-Ala-DAP (daughter metabolite), and 3-Ala-DAP (daughter metabolite).

the goal of understanding the underlying kinetic mechanism, which we expected could lead to the design of superior 3,4-DAP prodrugs.

Initially, we examined the stability of Bis-Ala-DAP in PBS buffer (pH 7.4) and observed no release of 3,4-DAP. However, upon incubation in mouse serum, the prodrug was readily hydrolyzed, likely mediated by peptidases present in serum. Mass spectral analysis and quantification as a function of incubation time revealed that both amide substituents are hydrolyzed, though there is considerably more buildup of 3-Ala-DAP than 4-Ala-DAP. Next, we examined each of the monosubstituted compounds' deamindation rates in serum. Figure 3a,d presents the conversion of 3-Ala-DAP to 3,4-DAP. The conversion is first order, and 3-Ala-DAP has a serum half-life of approximately 8 h. On the other hand, 4-Ala-DAP decomposed by two pathways, by deamidation to 3,4-DAP and by interconversion to 3-Ala-DAP (Figure 3b,e). Overall, the decomposition of 4-Ala-DAP in serum is also first order and has a half-life of approximately 1.7 h. By determining the decomposition pathways of the daughter metabolites, we could now assemble a complete kinetic picture of unmasking 3,4-DAP from Bis-Ala-DAP in mouse serum. To build a decomposition scheme consistent with our data, we began with Figure 3a determined from the study depicted in Figure 3d. From the study depicted in Figure 3e, we built up to Figure 3b, displaying the relationship between daughters 4-Ala-DAP and 3-Ala-DAP as well as unmasked 3,4-DAP. Lastly, a full pathway was constructed as shown in Figure 3c. Values for the individual rate constants are found in Table 1. As seen in Figure 3f, the kinetics of 3,4-DAP release from Bis-Ala-DAP in serum are well described by the proposed kinetic mechanism.

Table 1. Experimentally Determined Rate Constants for the Decomposition of Bis-Ala-DAP in Mouse Serum

rate constant	rate (h^{-1})
k_1	0.266 ± 0.12
k_2	2.21 ± 0.19
k_3	0.29 ± 0.035
k_4	0.083 ± 0.028
k_5	0.125 ± 0.024

From this data, several conclusions can be formulated: in mouse serum, there is an 8-fold preference for deamidation of the 3 position over the 4 position. Bis-Ala-DAP is consumed rapidly in a first order process with a half-life of approximately 17 min. 3-Ala-DAP is the major transitory metabolite being produced directly from Bis-Ala-DAP and indirectly by interconversion from 4-Ala-DAP. 3-Ala-DAP accounts for more than 60% of the total DAP species from between 0.5 and 5 h. On the other hand, 4-Ala-DAP never accounts for more than 12% of the total DAP concentration. Aside from a modest 30 min lag period, 3,4-DAP forms in a nearly first order process with an apparent formation half-life of 10 h.

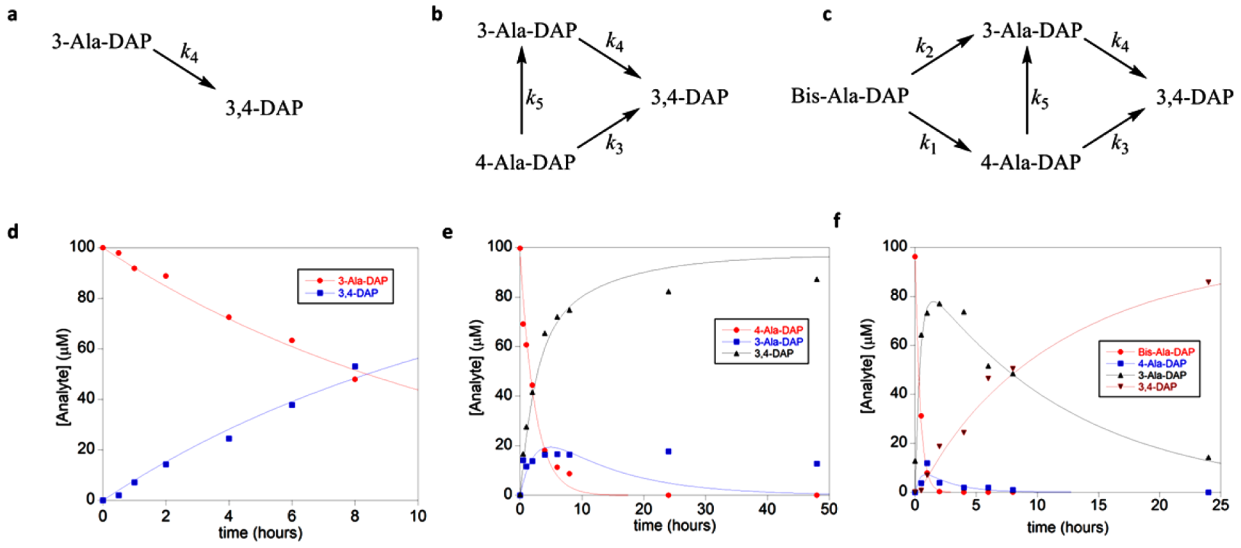

Figure 3. Decomposition pathways and kinetic plots for parent and daughter compounds in mouse serum as a function of time. (a) Decomposition pathway of 3-Ala-DAP to 3,4-DAP. (b) Decomposition pathway of 4-Ala-DAP to 3-Ala-DAP and 3,4-DAP. (c) Overall decomposition pathway of Bis-Ala-DAP to daughter metabolites and ultimately to 3,4-DAP. (d) Kinetic plot indicating conversion of 3-Ala-DAP to 3,4-DAP. (e) Kinetic plot indicating conversion of 4-Ala-DAP to 3-Ala-DAP and 3,4-DAP. (f) Overall kinetic plot indicating the decomposition of Bis-Ala-DAP to daughter metabolites and ultimately to 3,4-DAP.

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The decomposition pathway of Bis-Ala-DAP in mouse serum looked promising; as such, we examined the pharmacokinetics of Bis-Ala-DAP in mice. As a control, we also examined the pharmacokinetics of 3,4-DAP. Both compounds were dose-normalized to 6 mg/kg 3,4-DAP. 3,4-DAP and Bis-Ala-DAP are rapidly distributed in the mouse, affording PK profiles consistent with a one-compartment model. In vivo, release of 3,4-DAP from Bis-Ala-DAP is much more rapid than observed ex vivo in serum; in fact, parent and daughters approached LOQ at 15 min (our first time point). Our prodrug approach reduced the 3,4-DAP C_{max} and extended the half-life of 3,4-DAP but at the cost of proportionately lower exposure (Table 2). The major objective of our prodrug approach was to limit C_{max} by extending the half-life while maintaining AUC. Since we did not meet this objective, we did not evaluate the prodrug in our platform. While our prodrug strategy was unsuccessful, the potential of 3,4-DAP as an effective therapeutic antagonist has been demonstrated. Contrasting the pharmacokinetics of 3,4-DAP and Bis-Ala-DAP indicates that rapid in vivo clearance of these compounds remains a major obstacle. We posit that biochemical/synthetic polymers that allow temporal control over 3,4-DAP over an extended duration would be needed for maximum possible benefit from the aminopyridine's ability to alter intoxicated neuronal cells.[20]

Table 2. Pharmacokinetic Analysis of 3,4-DAP from 3,4-DAP or Bis-Ala-DAP Injection[a]

parameter	3,4-DAP	Bis-Ala-DAP
C_{max}	11	4.3
$t_{1/2}$	0.53	0.70
AUC	9.0	4.2

[a]Parameters reported as C_{max} (μM), $t_{1/2}$ (h), and AUC (μmol·h·L^{-1}).

CONCLUSIONS

Over the past 20 years, research initiatives to treat BoNT intoxication have failed.[5,6] While the sequence of events leading to the toxin pathology suggests several avenues for pharmacological intervention, none have succeeded. It can be reasoned that these failures are due to BoNT/A's extreme toxicity; however, we would argue that maybe more important is its persistence. Accordingly, even after a lethal quantum of toxin has entered nerve endings, the burden of toxin is still quite large. This translates to continued toxin accumulation at nerve endings; this in turn will lengthen the duration of intoxication. Given our findings, we submit it is illogical to adopt any therapeutic strategy that permits toxin circulation to serve as a reservoir from which toxin can continue to be delivered to nerve endings over an extended period of time. It also makes little sense to leave a neurotoxic agent with a long half-life in the body while trying to counteract it with an antagonist with a markedly shorter half-life. We have shown that a cogent intervention is a postchallenge administration of a sequestering agent to reduce toxin burden, and thereby prevent additional intraneuronal accumulation. The idea of reducing toxin load is important for pharmacologic antagonist intervention as we demonstrate with 3,4-DAP; it does not have to be fully optimized to provide a therapeutic efficacy when administered en masse. The value of our platform is that our findings can be used by any investigator or any company seeking to develop an antibody preparation or pharamacologic antagonist, in essence universal applicability.

METHODS

BoNT/A Lethality Assay. All mice used in this assay were female CD-1 mice (approximately 20 g, ca. 6 weeks old). Approximately 5 LD_{50} BoNT/A was used per mouse; this came from a type A neurotoxin standard that was titrated via quantal intraperitoneal bioassay using five dilutions having 30 mice per dilution. A volume of 0.5 mL of a solution containing 10 LD_{50}/mL was injected intraperitoneally. All antibodies used were equine IgG and were made by Metabiologics using purified type A neurotoxin as the immunogen. These antibodies have a neutralizing titer of 20 IU/mg. Titration was done using five dilutions of IgG with 10 animals per dilution and titrating against 10 000 LD_{50} of the type A neurotoxin standard described above. All antibodies were polyclonals and given intraperitoneally after 2 h of BoNT/A administration. After 3 h of BoNT/A administration, a PBS solution of 3,4-DAP was administered such that the effective concentration of 3,4-DAP was 10 mg/kg body weight (n = 4–6, per test group).

Prodrug and 3,4-DAP Analysis via LC/MS. All experiments were performed on a Agilent MSD 1100 Series electrospray ionization mass spectrometer (Column: Agilent Zorbax SB-CN, 1 × 150 mm, 3.5 μm). Solvent system: solvent A, H_2O; solvent B, 10% 100 mM NH_4OAc (aq)/90% MeCN. Dimethyl sulfoxide (DMSO; BioReagent grade), MeCN (LC/MS grade), MeOH (LC/MS grade), and mouse serum were purchased from Sigma Alrich. All data was analyzed using Agilent MassHunter Qualitative Analysis software. Individual calibration curves were prepared for each compound by determining the peak area from the respective extracted ion chromatograms and plotting against a known concentration.

3,4-DAP Release Studies in Mouse Serum. The appropriate compound was dissolved in DMSO to yield a 10 mM stock solution. A volume of 10 μL of DMSO stock solution was added to 1 mL of mouse serum and incubated at 37 °C with shaking. Next, 100 μL of the incubated solution was removed at the desired time points, and then added to 200 μL of MeCN containing 3,4,5-triaminopyridine[10] (10 μM) as an internal standard, and then centrifuged @ 14 000 rpm for 15 min. Finally, 50 μL of supernatant was then removed and analyzed via LC/MS.

3,4-DAP Release Studies in PBS Buffer (pH 7.4). The appropriate compound was dissolved in DMSO to yield a 10 mM stock solution. A volume of 10 μL of DMSO stock solution was added to 1 mL of PBS buffer and incubated at 37 °C with shaking. Next, 100 μL of the incubated solution was removed at the desired time points, and then added to 200 μL of MeCN containing 3,4,5-triaminopyridine[10] (10 μM) as an internal standard, and then centrifuged @ 14 000 rpm for 15 min. Finally, 50 μL of supernatant was then removed and analyzed via LC/MS.

Pharmacokinetics. Solutions (1:10 DMSO/saline) of 3,4-DAP and Bis-Ala-DAP were made to yield a final effective concentration of 6 mg/kg 3,4-DAP. Following injection, approximately 100 μL of blood was sampled from the tail vein at each time point (15, 60, 120, and 240 min) into heparinized eppendorf tubes. These were centrifuged, and the plasma was removed and transferred to fresh tubes and frozen until assayed. All samples were extracted from male mice, weighting approximately 30 g (n = 3 per compound tested).

ASSOCIATED CONTENT

Supporting Information

Representative extracted ion chromatograms, ^{1}H NMR spectra, ^{13}C NMR spectra, and integrated rate equations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

[§]T.L.H. and C.A.L. contributed equally to this work and were involved in all aspects of this study, including synthesis of compounds, LC/MS experiments, and data analysis.

M.S.H. was involved in the kinetic analysis of the prodrug decomposition pathway. K.D.J. conceived the project.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

3-Ala-DAP, (*S*)-2-amino-*N*-(4-aminopyridin-3-yl)propanamide; 3,4-DAP, 3,4-diaminopyridine; 4-Ala-DAP, (*S*)-2-amino-*N*-(3-aminopyridin-4-yl)propanamide; Ab, antibody; AUC, area under the curve; Bis-Ala-DAP, (2*S*,2′*S*)-*N*,*N*′-(pyridine-3,4-diyl)bis(2-aminopropanamide); BoNT, botulinum neurotoxin; CNS, central nervous system; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; FDA, Food and Drug Administration; HC, heavy-chain; LC, light-chain; LC/MS, liquid chromatography−mass spectroscopy; LD_{50}, median lethal dose; LOQ, limit of quantification; PBS, phosphate buffered saline; PK, pharmacokinetic; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

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