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Development and Evaluation of Botulinum Neurotoxin Therapeutics

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The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

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PART 1 - Development and Evaluation of BoNT Therapeutics in Rats

1. INTRODUCTION

1.1. Background.

The botulinum neurotoxins (BoNTs) consist of seven neurotoxic proteins (A–G) produced by immunologically discrete strains of the bacterium *Clostridium botulinum* and less frequently by *C. baratii, C. butyricum, and C. argentinense* (Habermann and Dreyer, 1986; Simpson, 2004). The neurotoxins are secreted initially as relatively inactive 150 kDa protoxins (range 140–167 kDa) surrounded by a complex of neurotoxin-associated proteins that protect BoNT from degradation in the gastrointestinal tract (Gu et al., 2012). The protoxins are subsequently cleaved to form the active dichain neurotoxin, consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) (DasGupta and Sugiyama, 1972). Cleavage occurs by action of clostridial proteases, or in non-proteolytic strains, by host proteases. Cleavage is incomplete for BoNT/B and BoNT/E, thus these serotypes must be activated by controlled trypsinization prior to use in assays (Simpson and Dasgupta, 1983; Wang et al., 2010) (See Materials and Methods section).

The BoNTs have three functional domains: binding, translocation, and catalytic. Binding is mediated by the C-terminal region of the heavy chain, which interacts with gangliosides and protein receptors located at high density on cholinergic nerve terminals. This is largely responsible for cholinergic nerve terminal being the preferred target of toxin action (Berntsson et al., 2013). The N-terminal region of the HC promotes the translocation of the LC near transmitter release sites in the nerve terminal cytosol, thereby facilitating the LC-mediated cleavage of soluble-N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins (Fischer and Montal, 2007).

1.2. Mechanism of Action.

The LC is a Zn^{2+}-dependent endopeptidase that cleaves specific sites on SNARE proteins that results in inhibition (and in high doses to cessation) of evoked transmitter release (Pirazzini et al., 2017). Since the interaction of SNARE proteins on the surface of synaptic vesicles and nerve terminal membranes are required for transmitter release in nearly all synapses, BoNT LCs have the inherent ability to inhibit synaptic transmission throughout the nervous system (Tighe and Schiavo, 2013). In actual outbreaks, however, the action of BoNT is restricted to inhibition of acetylcholine (ACh) release as a consequence of the selective binding of the HC to cholinergic nerve endings. The latter, in conjunction with the inability of BoNT to cross the blood brain barrier (Wong et al., 2013; Adler et al., 2022), confines the actions of BoNT to the neuromuscular junction, autonomic ganglia and parasympathetic nerve terminals, while largely sparing sensory networks and CNS function (Matak et al., 2016; Pirazzini et al., 2017).

In skeletal muscle, inhibition of transmitter release leads to flaccid paralysis, which can progress to generalized muscle weakness and death when the muscles of respiration become sufficiently compromised. Early signs of BoNT intoxication include visual disturbances, difficulties in swallowing and impairment of speech (Sobel, 2005). At this stage, botulism can be treated by infusion of serotype specific antitoxin, which may limit further progression. However, when symptoms advance to generalized paralysis and respiratory weakness, antitoxin

becomes less effective, and patients will need treatment in an intensive care facility (Tacket et al., 1984; Hatheway et al., 1984). The more severe cases may also require artificial ventilation, enteral feeding and physical therapy after discharge from intensive care (Shapiro et al., 1998; Robinson and Nahata, 2003; Marcus, 2009).

1.3. Medical Countermeasures for BoNT Intoxication.

Following the discovery that local injection of minute quantities of BoNT/A was effective for treatment of dystonia, movement disorders and other diseases stemming from cholinergic hyperactivity (as well as for aesthetic improvement) (Anandan and Jankovic, 2021), the research community has come to view BoNT less as an existential threat and more as a useful drug. Accordingly, the current interest in BoNT is overwhelmingly focused on the discovery of new medical and aesthetic indications for this toxin. Although adverse events (AEs) from BoNT injections do occur, they are rare and usually resolve without treatment (Omprakash and Rajendran, 2008; Jain et al., 2023).

The small number of natural outbreaks and AEs are insufficient to justify a costly medical countermeasures development program by pharmaceutical companies and non-DoD government agencies, especially in light of availability of antitoxin (despite its limited window for successful administration) (O'Horo et al., 2018). This is clearly evident by the low number of new publications on medical countermeasures for botulism in the current literature. A recent PubMed query of "botulinum toxin and movement disorders" returned 307 articles for the years 2020-2022, whereas queries for "medical countermeasures and botulinum intoxication" (or similar search terms) yielded only 6 relevant publications for the same 3-year period. Of these, the general focus was the development of a more effective and sustainable source of BoNT antitoxin.

During the last three decades, a number of approaches have been used to discover pharmacological antagonists for BoNT: these consist of small molecule inhibitors (SMIs) to inactivate the BoNT LC, strategies to enhance removal of the LC from intoxicated nerve terminals (e.g., deubiquitinase inhibitors) (Tsai et al., 2017), neuronal delivery of single chain camelid antibodies (nanobodies) using BoNT/X (Miyashita et al., 2021) or detoxified BoNT/D (McNutt et al., 2021), and physiological antagonists such as 3,4-diaminopyridine (3,4-DAP) and other K^+ channel blockers to restore ACh release, especially in BoNT/A-intoxicated nerve terminals (Adler et al., 2019).

To date, SMIs have only been able to slow the rate of BoNT-mediated paralysis, but most exhibited little or no cell-based or *in vivo* efficacy (Dickerson et al., 2014; Duplantier et al., 2016) (see however Jacobson et al., 2017). Strategies to accelerate the removal of the BoNT LC from the nerve terminal have not progressed beyond the proof-of-concept stage (Tsai et al., 2017) while neuronal delivery of nanobodies is still in early development and would likely face difficult path for regulatory approval. In contrast to these approaches, physiological antagonists such as the aminopyridines, whose efficacy was first demonstrated over 40 years ago, have shown remarkable promise both in slowing the onset of BoNT-mediated muscle paralysis and in restoring tension in BoNT/A-paralyzed muscles (Molgó et al., 1980, 1987; Adler et al., 1995, 1996; Mayorov et al., 2010; Vazquez-Cintron et al., 2020; Machamer et al., 2022). Among the aminopyridine compounds examined, 3,4-DAP proved to be the most potent and least toxic drug candidate.

1.4. 3,4-DAP as a Novel Medical Countermeasure for BoNT Intoxication.

In a previous study from our laboratory, 3,4-DAP was shown to counteract BoNT/A-induced muscle paralysis in isolated phrenic nerve-hemidiaphragm preparations (Adler et al., 1995). In this study, concentrations of 3,4-DAP above 2 µM were found to be effective in increasing muscle tension, and concentrations ≥100 µM were able to restore twitch tension to near control levels. However, subsequent *in situ* experiments with 3,4-DAP in the rat extensor digitorum longus (EDL) preparation revealed that the duration of action of 3,4-DAP was brief, lasting <3 h when given by i.v. injection (Adler et al., 1996). Molgó et al. (1980) demonstrated that the short duration of action of 3,4-DAP can be attributed to its rapid clearance and not to tachyphylaxis or desensitization. Indeed, Adler et al. (1996) found that 3,4-DAP can be injected up to eight times over a 10-hour interval with no loss of its ability to reverse muscle paralysis in rats locally injected with high doses of BoNT/A. Similar findings have also been reported by Vazquez-Cintron et al. (2020) in BoNT/A-intoxicated mice.

1.5. Proof of Concept for Efficacy of 3,4-DAP Infusion.

Since a major limitation to the clinical use of 3,4-DAP is its brief duration, a proof-of-concept study was performed using subcutaneous (s.c.) administration of this drug via implanted osmotic minipump infusion for 7 days in rats whose EDL muscles were paralyzed by a local injection of BoNT/A. Infusion of 3,4-DAP was found to protect EDL muscles from BoNT/A-induced muscle paralysis for the entire 7-day period of infusion (Adler et al., 2000). In contrast, in vehicle-infused rats, the same local injection of BoNT/A caused total muscle paralysis (Adler et al., 2000). Importantly, however, protection was only present during 3,4-DAP infusion, as demonstrated by the recurrence of paralysis following surgical removal of the 3,4-DAP-containing minipumps after day 7. Although this study suggested that 3,4-DAP infusion can provide sustained protection from the paralytic action of BoNT/A, it was not clear whether protection observed in a single locally intoxicated muscle would equate to increased survival following systemic BoNT/A exposure.

1.6. Current Study to Extend 3,4-DAP to Systemic BoNT Intoxication. To determine whether 3,4-DAP could protect rats against systemic botulism, we studied the actions of 3,4-DAP infusion in rats intoxicated with sub- and supra-lethal doses of BoNT using survival as the primary endpoint and prolongation of survival time and symptomatic relief as secondary endpoints. We also extended the infusion time from 7 days to 14 days since the benefits of 3,4-DAP on EDL muscle tension was not maintained when infusion was discontinued after 7 days. In addition, we studied the three serotypes (BoNT/A, BoNT/B and BoNT/E) implicated in >98% of human botulism outbreaks (Shapiro et al., 1998) and switched from implanted osmotic minipumps to external infusion pumps to provide more flexibility for adjusting 3,4-DAP doses and infusion times. In addition to the study on rats, we have also performed a preliminary study to evaluate the suitability of using rabbits as large animal models for carrying out pharmacokinetic and other advanced studies on 3,4-DAP. For the current effort, we have determined the LD_{50} of BoNT/A in New Zealand white rabbits using the animal conserving up-and-down method. The data on rabbits are presented as Part 2 of this report. The LD_{50} of BoNT/B and BoNT/E and protection studies using 3,4-DAP infusion in rabbits are expected to be done at a future time contingent on availability of funding.

2. MATERIALS AND METHODS

2.1 BoNT Stock and Working Solutions. BoNT/A, BoNT/B and BoNT/E were purchased from Metabiologics Inc. (Madison, WI). The parent vials of each serotype contained 100 µg of pure 150 kDa neurotoxin dissolved in 100 µL of aqueous solution buffered with 100 mM sodium phosphate and 50 mM sodium chloride (pH 7.4). To prepare stock solutions of BoNT/A, 5 µL of toxin from the parent vial was added to 495 µL of phosphate-buffered saline (PBS, MilliporeSigma, St. Louis, MO) containing 0.2% gelatin (gelatin phosphate buffer, GPB), dispensed in 10 µL aliquots and frozen at -80 ^{o}C until use. The BoNT/A concentration in each vial of stock solution was 10 ng/µL. Since BoNT/B and BoNT/E are only partially activated by bacterial proteases, they were fully activated by a controlled enzymatic digestion of an aliquot of toxin from the parent vial using TPCK-treated trypsin (MilliporeSigma) according to the method of Wang et al. (2015). Trypsin activation generally results in a 3-fold increase in BoNT/B activity and a more striking 100-fold increase for BoNT/E activity (Simpson and Dasgupta, 1983; Wang et al., 2010).

Prior to each experiment, one tube of this stock solution was further diluted with PBS containing 0.2% gelatin to obtain working concentrations suitable for toxicity testing. A separate vial of the 10 ng/µL stock solution was used for each experiment to avoid loss of activity from freeze-thaw damage.

2.2 Animals. The studies described in Part 1 of this Final Report were performed in mice and rats; a preliminary study to determine the LD_{50} of BoNT/A in rabbits will be described in Part 2 of this Final Report. Animals were housed in facilities approved by AAALAC International with food and water provided ad libitum. Animals were maintained in a temperature, light, and humidity-controlled environment in accordance with USAMRICD SOPs and the most recent edition (8^{th}) of the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011). A 12-hour light/dark, full-spectrum lighting cycle with no twilight were maintained in all animal holding areas. Mice and rats were quarantined for 5 days after arrival; they were group housed until the start of the study and individually housed after toxin exposure.

2.2.1. Determination of the intravenous (i.v.) BoNT LD_{50} values in mice. Because of the steep dose response curve for BoNT-mediated toxicity, small lot-to-lot variations in the toxin's specific activity can dramatically affect lethality (Pearce et al., 1994). Variations in potency in commercial preparations occur because BoNT/A is derived from bacterial cultures, and comprehensive potency testing may not be performed on each new lot of neurotoxin by the manufacturer; potency testing must therefore be performed by the user.

We determined the potency of BoNT/A, BoNT/B and BoNT/E using the mouse lethality assay on CD1 mice of either sex weighing 20-32 g (Charles River Laboratories). Mice were lightly anesthetized with an 5% isoflurane-oxygen mixture and injected in the tail vein with BoNT in a volume of 0.2 mL. Mice were weighed daily and observed twice per day for toxicity and mortality for 5 days after exposure. Typically, five doses of BoNT were tested using 4-6 mice per dose. The doses were selected to bracket Metabiologic's nominal LD_{50} and were incremented or decremented by factors of 1.5 (2 doses above and 2 below the nominal LD_{50}) as suggested by Pearce et al. (1994). The mean LD_{50} of BoNT/A, BoNT/B and BoNT/E in mice was determined to be 0.38, 0.49 and 0.84 ng toxin per kg, respectively, for the 3 BoNT serotypes. Mice were euthanized at the end of the 5-day study or earlier if they lost >35% body weight, exhibited acute respiratory distress or gross hematuria, or met combined endpoint scoring criteria for toxicity. Euthanasia was performed by a 5-min exposure to CO_2 followed by decapitation.

2.2.2. Determination of the i.v. BoNT LD_{50} values in rats. The i.v. LD50 of BoNT/A, BoNT/B and BoNT/E was determined in adult male Sprague Dawley rats (Charles River Laboratories) weighing 299-338 g at the beginning of the study. Rats were group housed prior to assignment to an experimental group and individually housed thereafter; in each case with free access to food and water. For determining the BoNT LD_{50}, rats were lightly anesthetized with an 5% isoflurane-oxygen mixture and injected in the tail vein with BoNT in a volume of 0.25 mL. Animals were allocated to 5 dosage groups, each containing 4-5 rats. For BoNT/A and BoNT/E, the mouse LD_{50}/kg body weight provided a provisional estimate of the rat i.v. LD_{50}, which made it possible to obtain the actual LD_{50} by using only 2 doses above and 2 below this estimated value. This was possible because mice and rats have similar sensitivity to BoNT/A and BoNT/E (Donald et al., 2018).

A somewhat different procedure was required for BoNT/B, since rats are known to be considerably less sensitive than mice to this serotype. Burgen et al. (1949) first reported this

species difference and estimated its order of magnitude to be approximately 500-fold, which was confirmed by subsequent authors (Adler et al., 1996; Elliott et al., 2017). Using this value as a guide, LD_{50} studies were carried out with doses of BoNT/B ranging from 300 to 900 mouse LD_{50} units.

After exposure, rats were weighed daily and observed twice per day for toxicity and mortality for 7 days. Rats were humanely euthanized at the end of the study or earlier if they lost >35% body weight, exhibited acute respiratory distress or manifested gross hematuria. Euthanasia was performed by intraperitoneal (i.p.) injection of pentobarbital (200 mg/kg) followed by thoracotomy.

2.3. Evaluation of 3,4-DAP for Treatment of BoNT Toxicity

2.3.1. Infusion parameters.

To carry out infusion of vehicle or 3,4-DAP (MilliporeSigma), rats were implanted with subcutaneous (s.c.) catheters by Charles River Laboratories via incision in the upper back and insertion of the catheter into a dorsal tunnel, with the infusion port secured between the scapulae. Catheters were flushed every 3–5 days with 0.5 mL saline to ensure patency. The surgically implanted infusion port was stabilized with a rat harness (VAH95AB, Instech Laboratories, Plymouth Meeting, PA) to prevent accidental removal and connected to a tether kit, which ran from the infusion port through a counterbalance lever arm to 10 mL plastic syringes containing vehicle (saline) or 3,4-DAP dissolved in saline. Syringes were controlled with Pump 11 Elite syringe pumps (Harvard Apparatus, Holliston, MA). Tethers were filled with treatment solution prior to connection to the infusion port. Rats were acclimated to the infusion harness 1 day before intoxication.

2.3.2. Delivery of 3,4-DAP or vehicle in catheterized rats.

Anesthetized adult male rats were injected intravenously (i.v.) with BoNT/A, BoNT/B or BoNT/E in the tail vein, and 12 or 24 h later, 3,4-DAP or vehicle was infused s.c. via surgically implanted catheters at constant rates of 0.33 µL/h. Allocation of rats to vehicle or 3,4-DAP treatment groups was randomized. Two infusion doses were used: 0.98 mg/kg.h and 1.44 mg/kg.h, generating steady-state plasma 3,4-DAP concentrations of 125 and 200 ng/mL, respectively. At the start of 3,4-DAP infusion, rats also received a bolus intramuscular (i.m.) injection of 1 mg/kg 3,4-DAP as a loading dose, since the infused 3,4-DAP required 2 h to traverse the tubing and reach the animals. Rats were observed for survival and signs of botulism twice per day for two weeks and for an additional week following termination of infusion.

Body weights were determined daily. Eight specific signs of botulism were used for computing a Clinical Severity (CS) score. The aggregate CS score and survival were used as measures of efficacy of 3,4-DAP treatment. The CS score was based on findings of lethargy, porphyrin, limb weakness, “wasp-waist,” forced abdominal breathing, urinary retention, hematuria and total body paralysis. These signs were assigned weights of 1-3 depending on their prognosis for mortality. The CS score ranged from 0 for an asymptomatic rat to 16 if all 8 signs were observed. Rats exhibiting gross hematuria, gasping or profound loss of body weight (>35%) were assigned a value of 16 and humanely euthanized.

2.4. Data Analysis. Unless stated otherwise, data were expressed as mean ± SEM. The number of rats at the beginning of each experiment was equal (or nearly equal) for vehicle and 3,4-DAP and generally consisted of 6 or more animals. Data analysis was performed using GraphPad Prism v9.4.1. LD_{50} values were derived from non-linear regression fits of log dose-lethality data. Survival data were plotted as Kaplan-Meier curves and differences between vehicle- and 3,4-DAP-infused rats were analyzed by Mantel-Cox log-rank test, with $P \leq 0.05$ considered to be statistically significant.

3-5. RESULTS

3. Protection by 3,4-DAP Against Intoxication by BoNT/A

3.1. Protection Against a 1 LD_{50} BoNT/A Challenge. Twelve rats were injected i.v. with a 1 LD_{50} dose of BoNT/A. At ~24 h after toxin challenge, which coincided with initial signs of botulism in rats (lethargy, porphyrin staining of eyes, hind limb weakness), rats were infused s.c. with vehicle (n= 6) or 3,4-DAP (n= 6). The 3,4-DAP-infusate had a concentration of 0.98 mg 3,4-DAP/kg body weight and was delivered by an external perfusion pump at a steady rate of 33 µl/h (8 mL per day) for 14 days via surgically attached s.c.-implanted catheters. These infusion parameters generated a steady-state 3,4-DAP plasma concentration of 125 ng/mL, as determined by earlier pharmacokinetic (PK) studies (Machamer et al., 2022). To compensate for the delay between onset of infusion and drug delivery, rats in the 3,4-DAP group also received a bolus injection of 1 mg/kg 3,4-DAP i.m. as a loading dose prior to onset of infusion.

3.1.1. Clinical severity (CS) score. Beginning at day 1, both vehicle- and 3,4-DAP-infused rats exhibited increasing signs of botulism, with clinical severity (CS) scores reaching 6 on day 3 (Figure 1A). Thereafter, the 3,4-DAP-infused rats began to recover, with CS scores decreasing to 5 at day 7, 2.7 at day 14 and 0 from day 15 to the end of the study. In vehicle-infused rats, CS scores continued to increase, reaching 10.6 on day 5, before returning to 6.0 for the remainder of the infusion period. The large reduction in CS values in vehicle infused rats between day 6 and 7 reflects the mortality of the most symptomatic rats, in conjunction with stabilization of signs in the 3-surviving vehicle-infused animals. The latter is more apparent in Figure 1B where CS data of only surviving rats are shown. It is clear from Figure 1B that in vehicle-infused rats, toxic signs from a 1 LD_{50} dose of BoNT/A do not begin to reverse until after day 14.

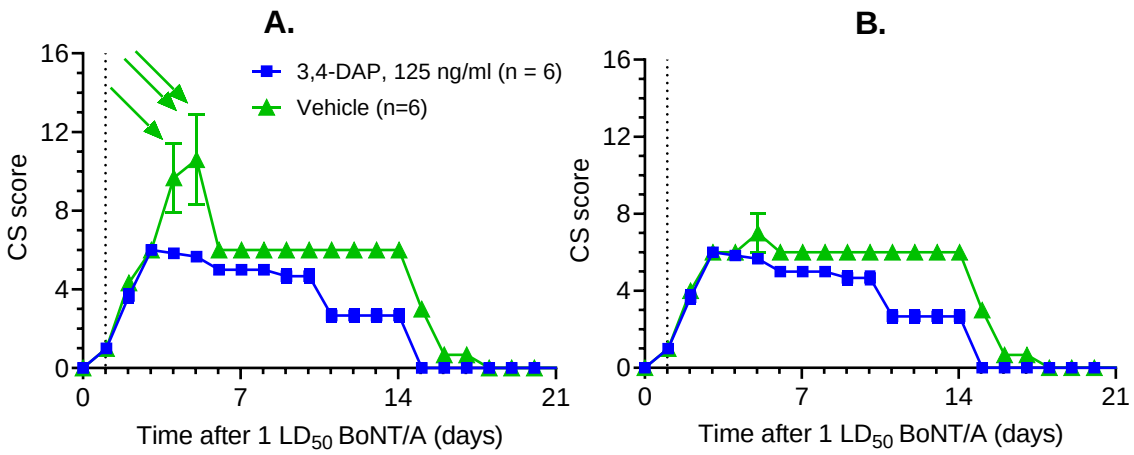

Figure 1. A. CS scores of rats challenged with a 1 LD_{50} dose of BoNT/A and infused with 0.98 mg/kg.h 3,4-DAP (125 ng/mL) for 14 days, beginning 1 day after toxin challenge. This infusion dose yielded a 3,4-DAP plasma level of 125 ng/mL as determined in prior PK studies (Machamer et al., 2022). Vehicle-infused rats exhibited signs of botulism of increasing severity for 5 days after challenge, followed by a plateau for the next 8 days and recovery thereafter. BoNT/A was injected in the tail vein at 0-time. The dotted line represents the onset of 3,4-DAP or vehicle infusion. The CS was based on 8 signs of botulinum intoxication encompassing lethargy, gait abnormalities, muscle weakness, apnea and recumbency (see Materials and Methods section 2.3.2). The CS scale ranged from 0 (asymptomatic) to 16 (found dead or meeting endpoint criteria and humanely euthanized- usually as a result of respiratory distress or urinary blockage). Differences in CS score between vehicle- and 3,4-DAP-infused rats were significant on post exposure days 4 and 5 ($P \leq 0.05$, Student's t-test). The arrows indicate time of mortality of 3 of 6 vehicle-infused rats. **B.** CS scores of surviving rats which encompasses all 3,4-DAP-infused rats and 3 of the 6 vehicle-infused rats.

3.1.2. Survival. The effect of 3,4-DAP on survival is shown in Figure 2. In vehicle-infused rats, a 1 LD50 dose of BoNT/A led to the mortality in 3 of 6 rats: one animal on day 4 and two on day 5. The three-remaining vehicle-infused rats and all six 3,4-DAP-infused rats survived the 2-week infusion period as well as the 1-week post-infusion period.

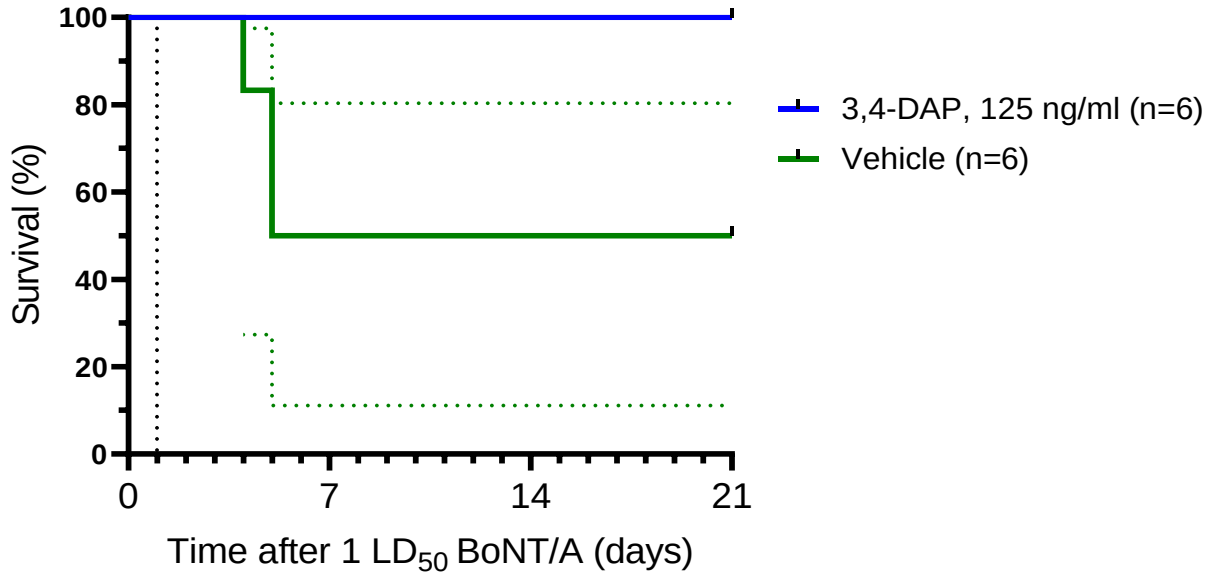

Figure 2. Survival of rats challenged with a 1 LD_{50} dose of BoNT/A and infused with vehicle or 0.98 mg/kg.h 3,4-DAP (125 ng/mL). Three vehicle-infused rats succumbed during the first week of infusion, but no deaths were observed in the 3,4-DAP group. BoNT was injected at 0-time; 3,4-DAP infusion was started 1 day after intoxication indicated by the vertical dotted line. Protection by 3,4-DAP was statistically significant (P <0.05; Mantel-Cox test). Green dotted lines indicate 95% CI.

3.1.3. Body weight. Figure 3 shows the changes in body weight in 3,4-DAP- and vehicle-infused rats. For the first 10-days after the 1 LD_{50} BoNT/A challenge, both groups underwent a profound body weight loss. This was due to a combination of factors which likely includes paralysis of the GI tract, difficulty in swallowing and extensive muscle weakness (Fan et al., 2016). Body weights began to recover at day 11 in the-3,4-DAP-infused rats, although these early gains were not maintained (Figure 3). In vehicle-infused rats, recovery of body weight did not begin until day 16. Interestingly, at the end of the 21-day observation period, the body weights of vehicle- and 3,4-DAP-infused rats no longer showed any difference.

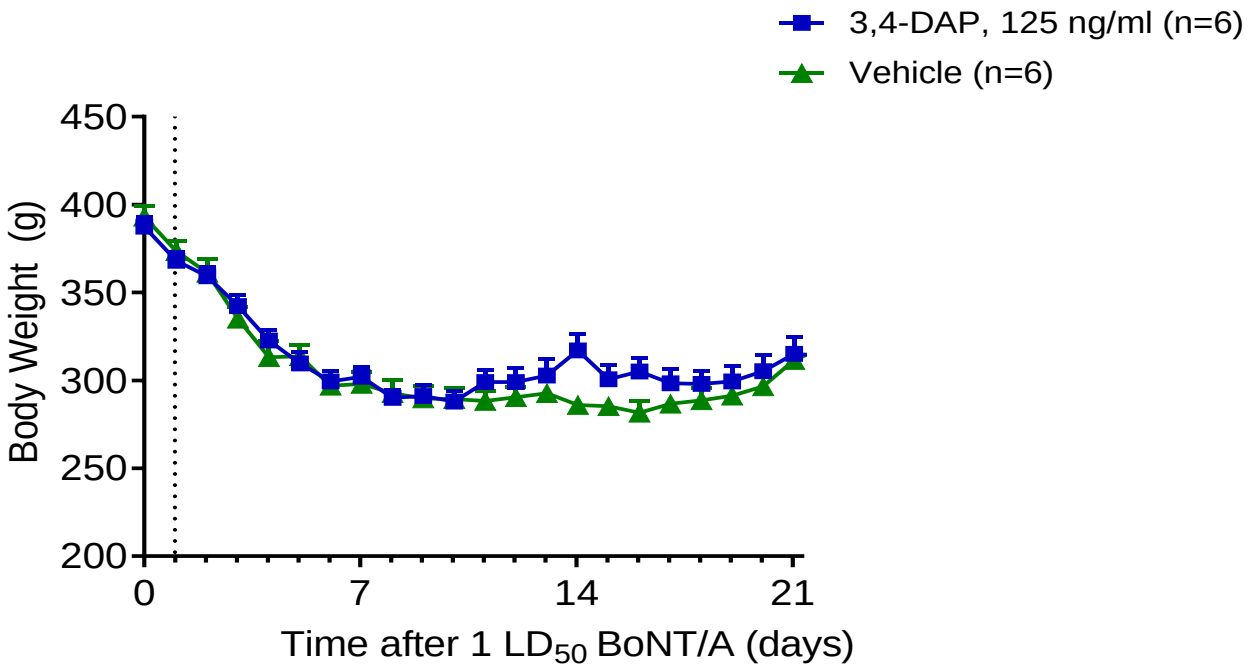

Figure 3. Body weights of rats challenged with a 1 LD_{50} dose of BoNT/A (0-time) and infused 1 day later (dotted line) with vehicle or 3,4-DAP. Maximum weight loss in vehicle- and 3,4-DAP-infused rats was 28.5 ± 1.7% and 25.7± 1.5 %, respectively.

3.2. Protection Against a 2.5 LD_{50} BoNT/A Challenge. The effect of 3,4-DAP infusion on CS score after intoxication by a 2.5 LD_{50} dose of BoNT/A is shown in Figure 4. As in the previous experiment, BoNT/A was administered by a single i.v. injection in the tail vein, and 3,4-DAP or vehicle was infused over a 14-day period. In this and all subsequent experiments (unless noted otherwise), the dose of 3,4-DAP was increased to 1.44 mg/kg.h, which resulted in steady-state 3,4-DAP plasma levels of 200 ng/mL as determined by prior PK studies. This is at the upper end of a human therapeutic plasma level of 3,4-DAP used in the treatment of Lambert-Eaton Myasthenic Syndrome (LEMS) and is well tolerated by patients (Thakkar et al., 2017).

3.2.1. CS score. As with the 1 LD_{50} BoNT/A challenge, signs and symptoms of botulism progressed in parallel in both vehicle- and 3,4-DAP infused rats for the first 2 days after exposure. Subsequently, signs and symptoms in the vehicle-infused rats became more severe until all vehicle-infused animals succumbed to the lethal actions of BoNT/A by day 6. In 3,4-DAP-infused rats, CS scores increased only slightly in severity beyond day 2, then reached a plateau of 5.3 from days 3 to 7. This was followed by a gradual recovery over the next 6 days, culminating in the absence of CS signs at the end of infusion on day 14. Importantly, 3,4-DAP-infused rats remained free of adverse signs during the 1-week post-infusion period with no mortalities or rebound toxicity.

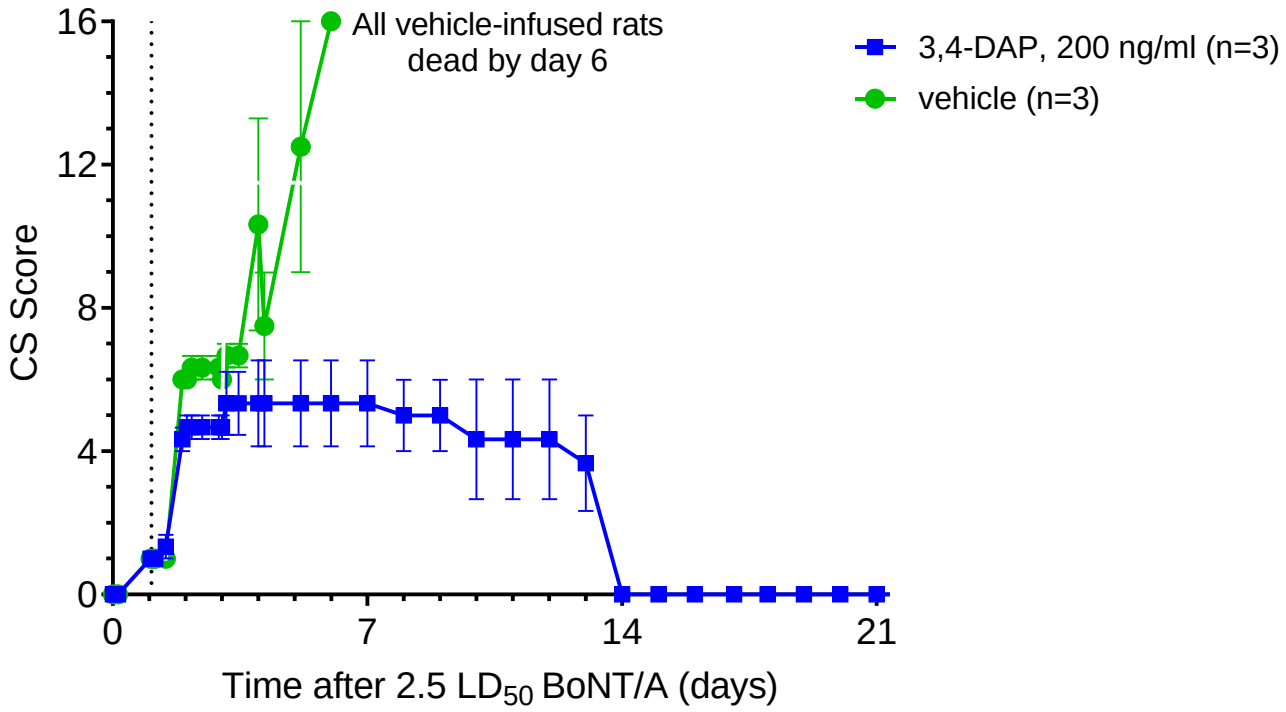

Figure 4. CS scores of rats challenged with a 2.5 LD_{50} dose of BoNT/A and infused with 200 ng/mL 3,4-DAP (as determined in a prior PK study) for 14 days, beginning 1 day after challenge. Note that both vehicle- and 3,4-DAP-infused rats exhibited signs of botulism of increasing severity during the first 2 days after challenge. BoNT/A was injected in the tail vein at 0-time and the dotted line represents the onset of 3,4-DAP- or vehicle infusion. Multiple CS observations are plotted on days 0 to 4 and 1 observation per day, thereafter.

3.2.2. Survival. Fourteen days of infusion with 3,4-DAP was found to be fully protective against a 2.5 LD_{50} challenge of BoNT/A as shown in Figure 5. Interestingly, if infusion of 3,4-DAP was terminated at day 5, all rats succumbed within the next day (not shown). These data indicate that the duration of infusion must be >5 days for survival, but we have not determined as yet whether the entire 14 days are required for survival.

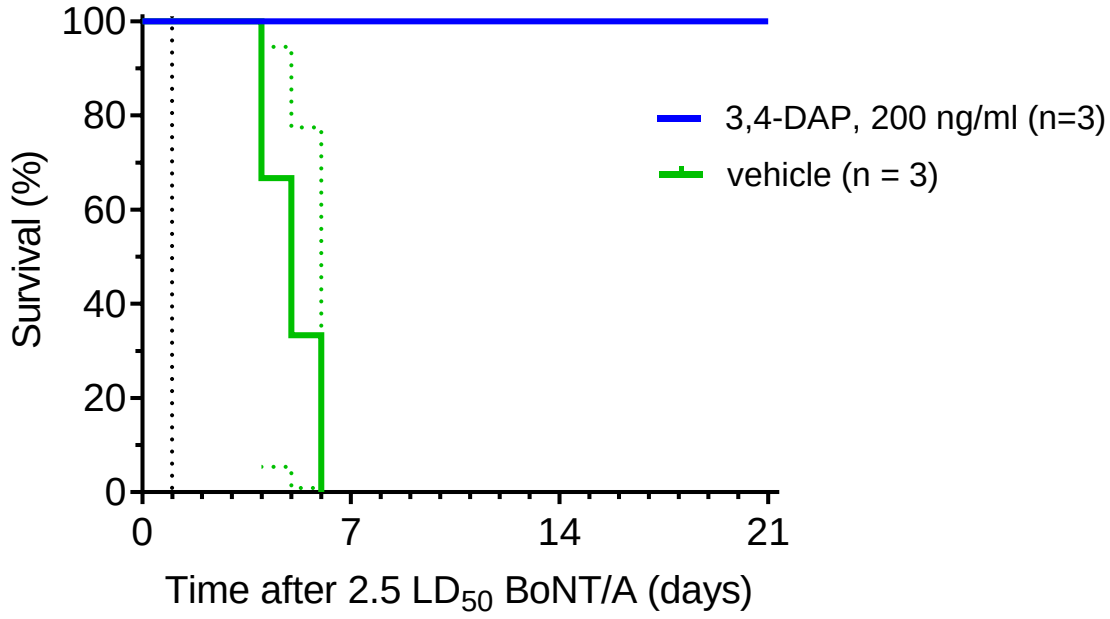

Figure 5. Survival of rats challenged with a 2.5 LD_{50} dose of BoNT/A and infused with vehicle or 3,4-DAP 24-h later. At this challenge dose, 200 ng/mL 3,4-DAP was fully protective, whereas vehicle-infused rats succumbed 4-6 days after challenge (median time of mortality = 5 days). Protection by 3,4-DAP was statistically significant (P=0.02; Mantel-Cox test). The green dotted lines indicate 95% CI.

3.2.3. Body weight. The effects of 3,4-DAP on body weight are shown in Figure 6. As with the 1 LD_{50} dose of BoNT/A, body weights decreased in parallel in vehicle- and 3,4-DAP- infused rats. Monitoring of the former was only possible for 5 days, since all vehicle treated rats succumbed by day 6. In spite of the treatment with 3,4-DAP, there was a substantial loss of body weight of >30%, due to extensive muscle weakness, difficulty swallowing and paralysis of the GI tract subsequent to BoNT/A challenge. The loss of body weight in 2.5 LD_{50} BoNT/A- challenged rats was even more profound than that observed in 1 LD_{50} challenged rats (compare with Figure 3).

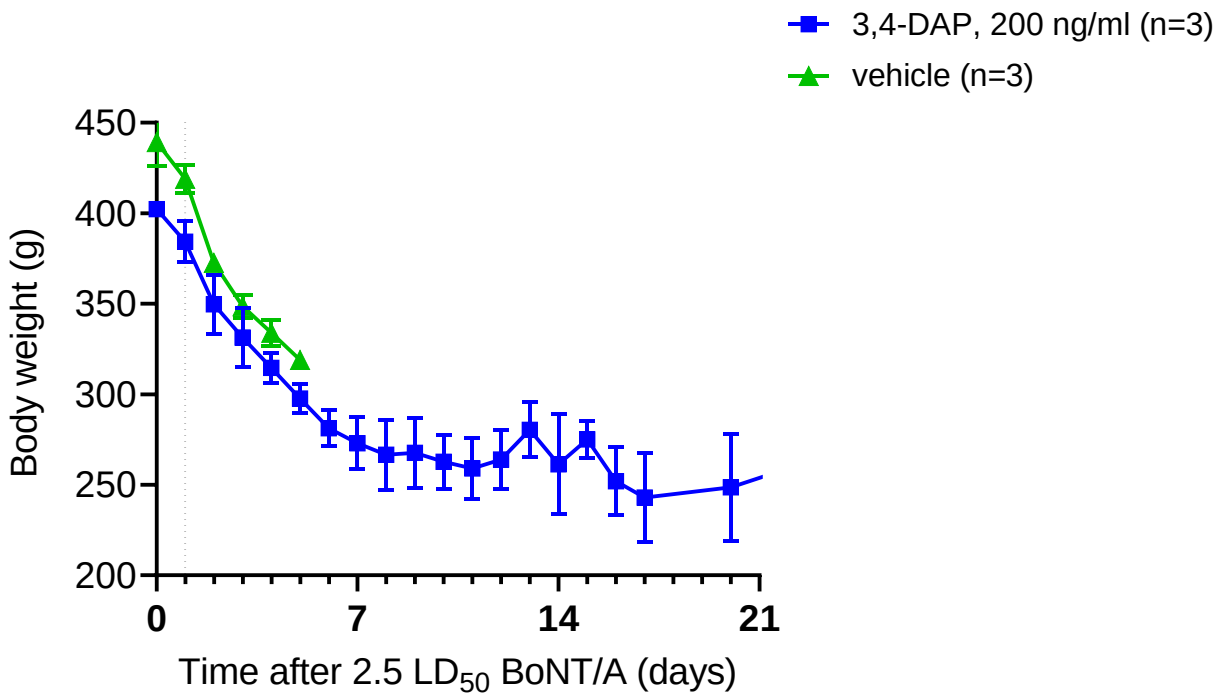

Figure 6. Body weights of rats challenged with a 2.5 LD_{50} dose of BoNT/A and infused 1 day later (dotted line) with vehicle or 200 ng/mL 3,4-DAP.

3.3. Protection Against a 4 LD_{50} BoNT/A Challenge-Trial 1: 3,4-DAP infusion dose of 0.98 mg/kg.h (125 ng/mL).

Two doses (125 ng/mL and 200 ng/mL) were used to test the efficacy of 3,4-DAP against a 4 LD50 challenge dose of BoNT/A. These experiments were carried out under similar experimental conditions as those used for the 1 and 2.5 LD_{50} studies, except for the earlier start of 3,4-DAP infusion (12 h rather than 24 h) due to the more rapid onset of clinical signs.

3.3.1. CS score.

At this high challenge dose of BoNT/A, signs of botulism were observed within 12 h of exposure and all vehicle-infused rats succumbed within 2 days of intoxication. Rats infused with 3,4-DAP at 0.98 mg/kg.h (125 ng/mL) had less severe signs (Figure 7) and survived for up to 6 days after challenge (Figure 8). However, none of the animals survived for the entire 2-week infusion period. Four of 6 rats were found dead during CS checks and 2 were humanely euthanized since they exhibited widespread paralysis and respiratory distress.

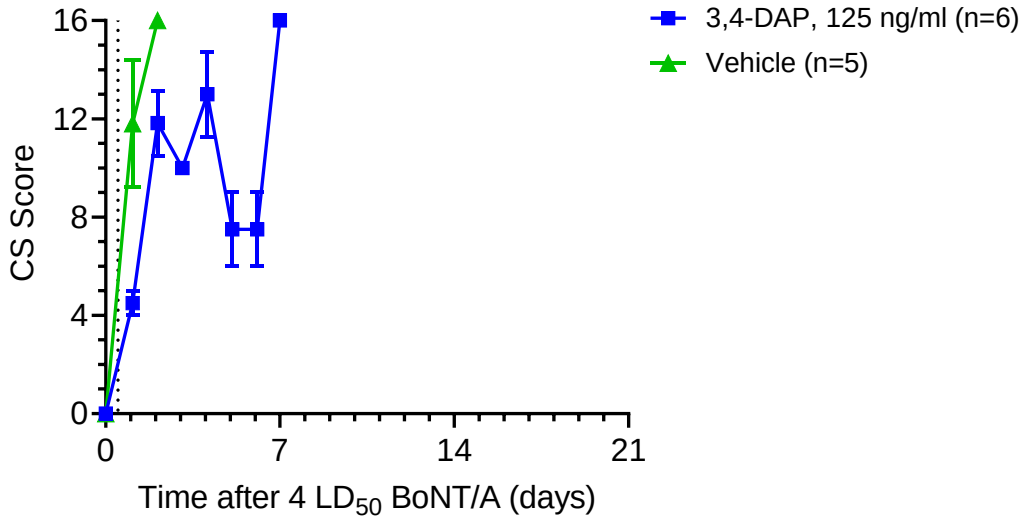

Figure 7. CS score of rats challenged with a 4 LD_{50} dose of BoNT/A and infused with vehicle or 125 ng/mL 3,4-DAP (as determined in a prior PK study). Infusion was started at the first signs of botulism at 12 h after BoNT/A challenge (lethargy, porphyrin staining of eyes, hind limb weakness). 3,4-DAP infusion delayed but did not arrest the severity of botulism (Compare with Figures 1 and 4).

3.3.2. Survival. The effect of 3,4-DAP (125 ng/mL) on survival is shown in Figure 8. At 4 LD_{50} BoNT/A, 3 of 5 vehicle-infused rats succumbed within 1 day of exposure and the remaining rats died by the next day. 3,4-DAP shifted the median survival time from 1 to 3.5 days, which is remarkable considering the extreme illness produced by 4 LD_{50} BoNT/A.

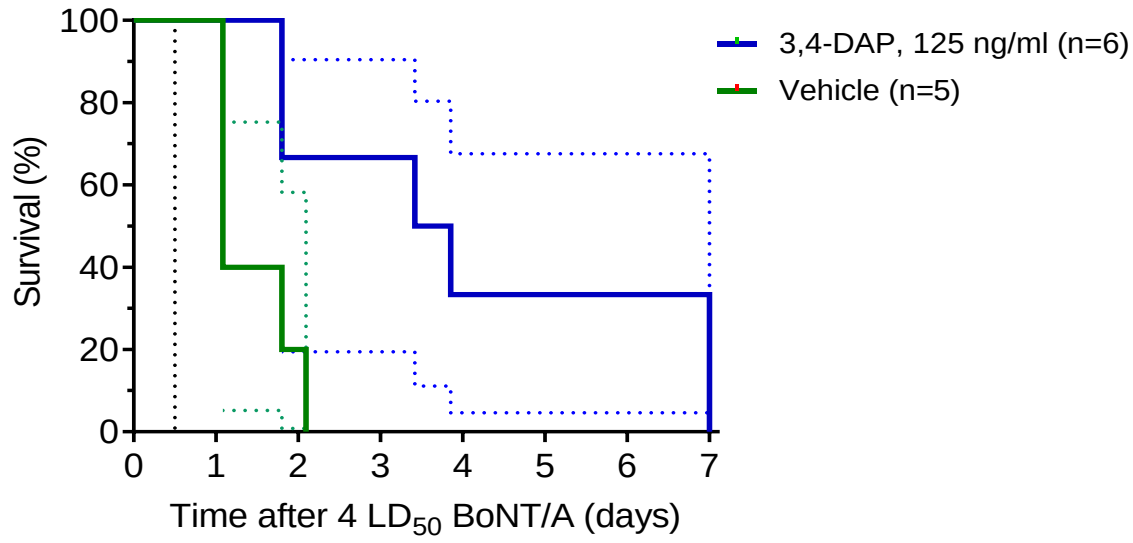

Figure 8. Survival of rats challenged with a 4 LD_{50} dose of BoNT/A and infused with vehicle or 125 ng/mL 3,4-DAP. At this challenge dose, 3,4-DAP prolonged the time to death, but did not protect against BoNT/A-mediated lethality. The median survival times were increased from 1 day in the vehicle-infused

group to 3.6 days in the 3,4-DAP infused group (P = 0.01; Mantel-Cox test). The green and blue dotted lines indicate 95% CI.

3.3.3. Body weight.

The effect of 3,4-DAP on BoNT/A-mediated loss of body weight is shown in Figure 9. At the infusion dose used, 3,4-DAP did not prevent weight loss, as indicated by the similarities in the rates of decline in body weight in vehicle- and 3,4-DAP-infused rats. Maximum weight loss of surviving 3,4-DAP-infused rats occurred on day 5, with mean body weights decreasing by 36% relative to values in this group one day prior to BoNT/A exposure (time 0).

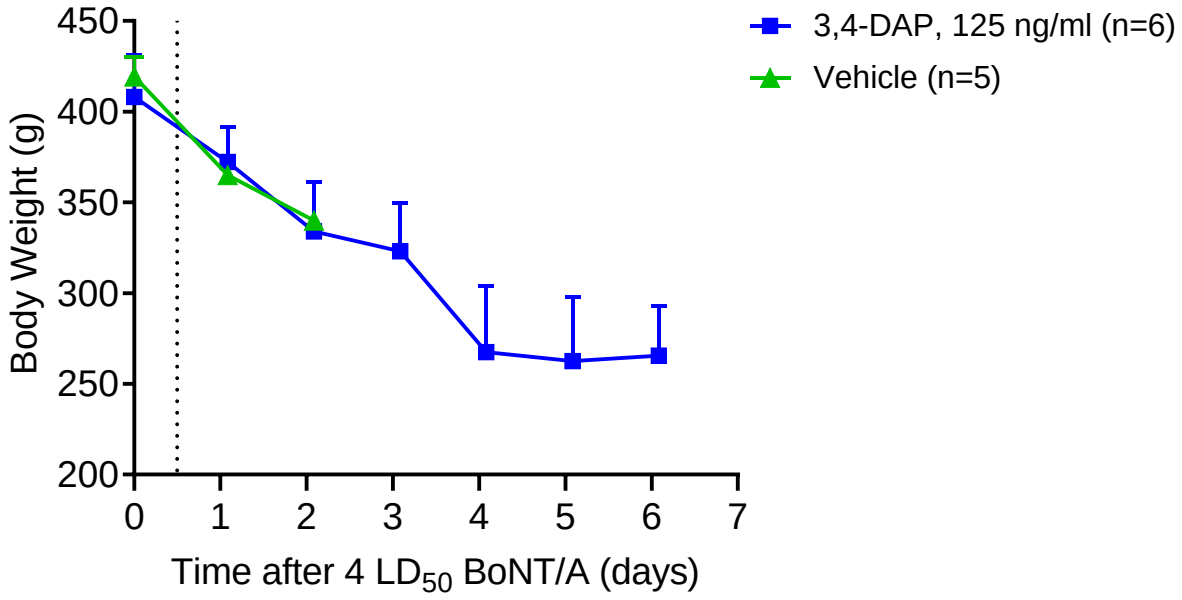

Figure 9. Body weights of rats challenged with a 4 LD_{50} dose of BoNT/A and infused 12 h later (dotted line) with vehicle or 3,4-DAP (125 ng/mL). Note that data for the former were plotted only for the first 2 days after exposure since no vehicle-infused rat survived beyond 2 days.

3.4. Protection Against a 4 LD_{50} BoNT/A Challenge-Trial 2: 3,4-DAP infusion dose of 1.44 mg/kg.h (200 ng/mL).

3.4.1. CS score, survival, and body weight. A second group of rats was also challenged with 4 LD_{50} BoNT/A, but this cohort was treated with 200 ng/mL 3,4-DAP instead of 125 ng/mL used in the first trial. Increasing the dose of 3,4-DAP resulted in improved CS scores (Figure 10) and longer survival times (Figure 11). An encouraging finding at the 200 ng/mL dose of 3,4-DAP was that one rat survived the entire 14-day infusion period plus the 7-day post-infusion time. Profound diaphragmatic weakness appears to play a major role in the high mortality seen with the 4 LD_{50} challenge dose of BoNT/A, especially during the first days after challenge. Autonomic abnormalities, such as paralysis of the GI tract, coupled with constipation and urinary retention also contribute to mortality, especially during later times. The marked improvement in all measured parameters with the 200 ng/mL 3,4-DAP infusion dose suggests that it would be of benefit to examine 3,4-DAP doses above those used in the present study, especially for treatment of intoxication by BoNT/A at 4 LD_{50} or higher.

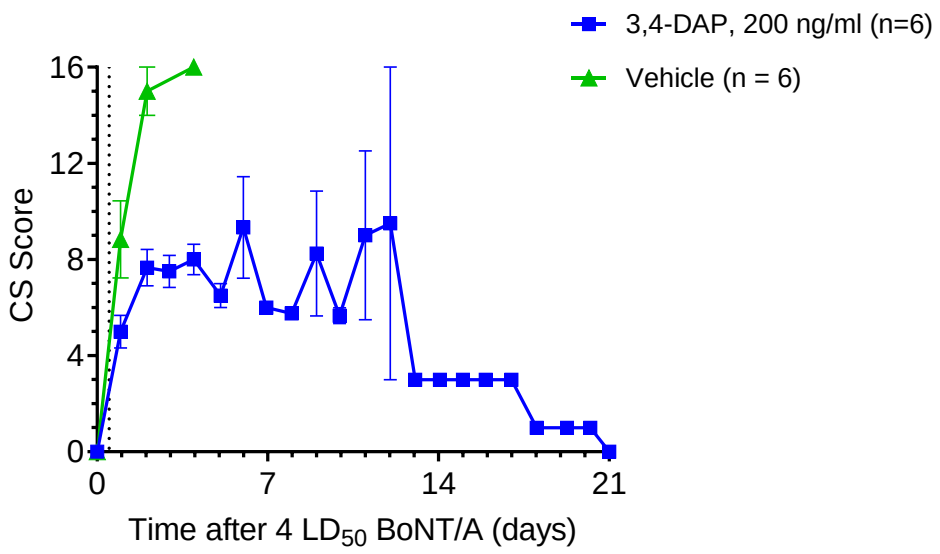

Figure 10. CS Scores of rats challenged with a 4 LD_{50} dose of BoNT/A and infused with vehicle or 200 ng/mL 3,4-DAP (as determined in a prior PK study). Infusion was started at 12 h which coincided with the first signs of botulism (e.g., porphyrin staining of eyes, lethargy, hind limb weakness). At 200 ng/mL, 3,4-DAP delayed the progression of CS signs and reduced their severity. It should be noted that the data from days 12 to 21 represents the CS score of one 3,4-DAP-infused rat that survived the 4 LD_{50} BoNT/A challenge.

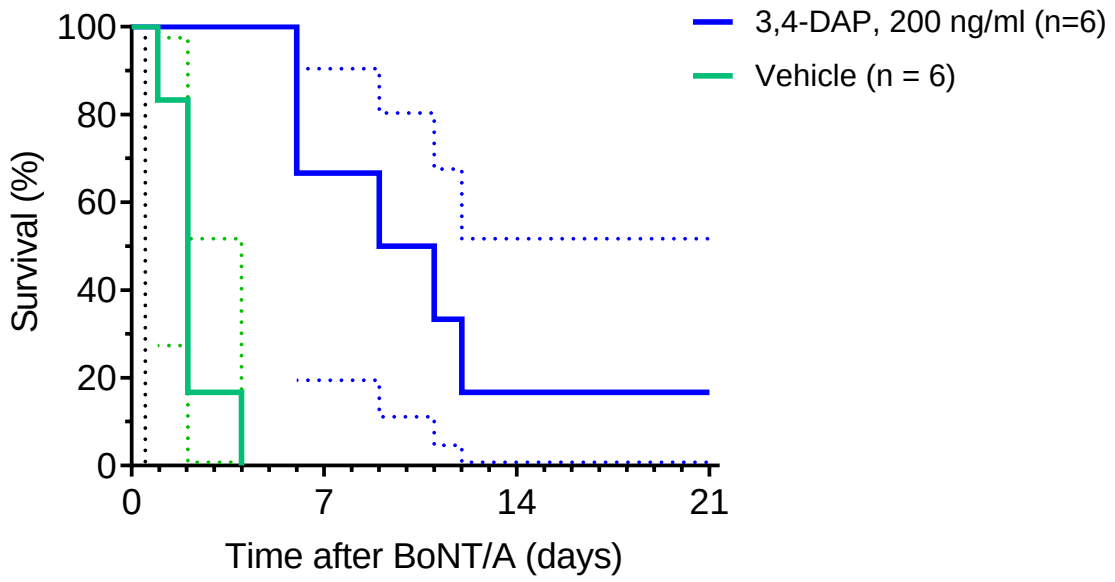

Figure 11. Survival of rats challenged with a 4 LD_{50} dose of BoNT/A and infused with vehicle or 3,4-DAP (200 ng/mL). The median survival time was 2 days in vehicle-infused rats and 10 days in 3,4-DAP-infused rats (P = 0.0006; Mantel-Cox test). The green and blue dotted lines indicate 95% CI.

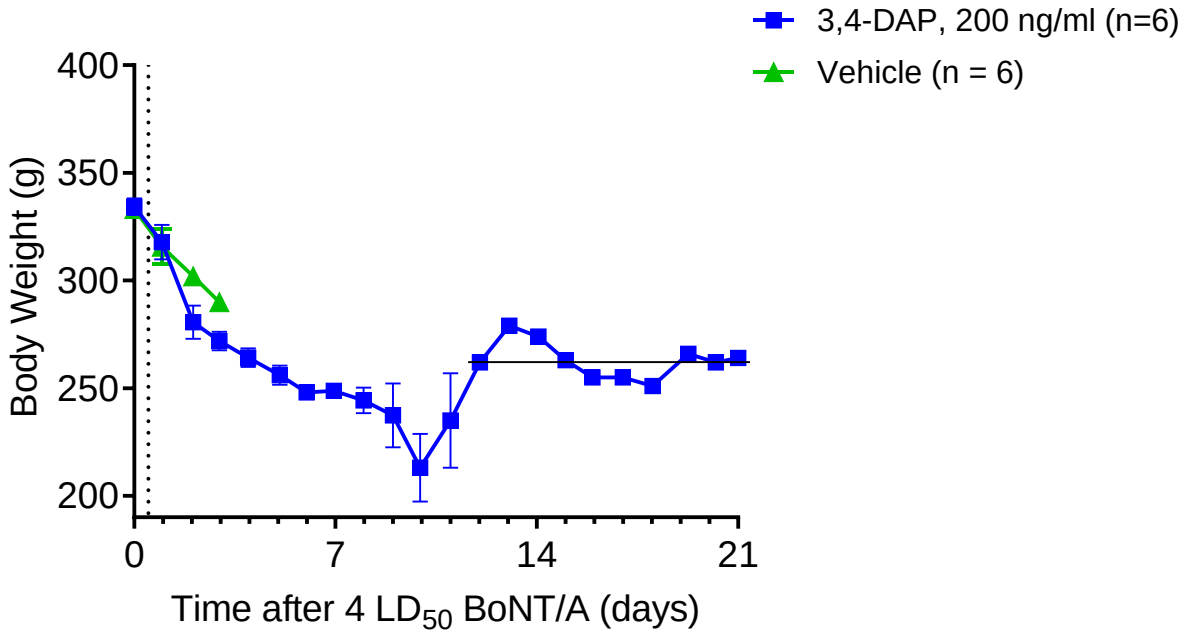

Figure 12. Body weights of rats challenged with a 4 LD_{50} dose of BoNT/A and infused 12 hours later with vehicle or 3,4-DAP (200 ng/mL). All vehicle-infused rats succumbed by day 3. Loss of body weight of up to 36% was observed on day 10 in the 3,4-DAP-infused group, which was the same as that observed in rats infused with the lower dose of 3,4-DAP (125 ng/mL; Figure 9). The apparent increase in mean body

weight after day 10 was due to the removal of 2 highly emaciated rats from the population under study. Data from day 12 to 21 reflects day-to-day fluctuation in the body weight of one surviving rat. It is clear from the horizontal line that there was no net increase in body weight over the interval shown.

4. Protection by 3,4-DAP Against Intoxication by BoNT/B.

Selection of an appropriate challenge dose for BoNT/B is difficult because rats are much less sensitive to this serotype than either mice or humans (Adler et al., 1996). Moreover, BoNT/B (and BoNT/E) require controlled activation by trypsin to generate the active dichain form of the toxin. We began testing BoNT/B in mice by tail vein injection to determine the potency of the activated stock solution. We then performed tests in standard non-catheterized rats by increasing the doses at multiples of the mouse LD_{50}. The LD_{50} for BoNT/B in rats was found to be 800 times the mouse LD_{50} when normalized for differences in body weight. This challenge dose was used subsequently in catheterized rats for determining efficacy of 3,4-DAP on BoNT/B.

4.1. Protection Against a 0.4 LD_{50} BoNT/B Challenge.

4.1.1. CS score.

The effect of 3,4-DAP infusion on CS score after intoxication by a 0.4 LD_{50} dose of BoNT/B is shown in Figure 13. BoNT/B was administered by a single i.v. injection in the tail vein, and 3,4-DAP (n=6) or vehicle (n=6) was infused s.c. via surgically implanted catheters over a 14-day period followed by a 7-day post infusion period. Signs characteristic of muscle paralysis (e.g., hindlimb weakness, impaired locomotion) typically appeared 2-3 days after i.v. injection of BoNT/B and increased in severity over the next 3-4 days (Figure 13). Intoxication progressed at similar rates for the first 3 days in both 3,4-DAP- and vehicle-infused rats, culminating in peak CS values of 6.6 and 10.1, respectively. Recovery occurred after day 4 in 3,4-DAP-infused rats but not until day 6 in vehicle-infused rats. In principle, a slower onset is advantageous for therapy by 3,4-DAP, since it provides more time to initiate treatment.

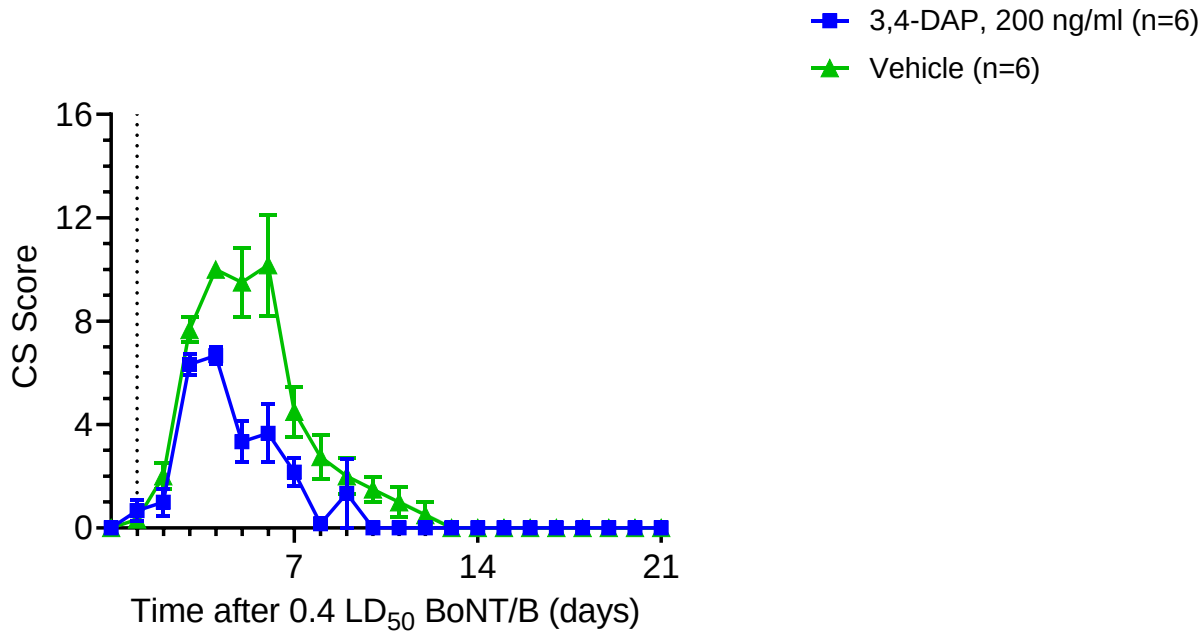

Figure 13. CS scores of rats challenged with a 0.4 LD_{50} dose of BoNT/B and infused for 14 days with vehicle of 200 ng/mL 3,4-DAP (as determined in a prior PK study). Infusion was started at 24 h after BoNT/B challenge.

4.1.2. Survival. At this low dose, only two vehicle infused rats and one 3,4-DAP infused rat died and, in both groups, mortalities occurred near the middle of the infusion cycle (Figure 14). The differences in the two survival curves were not statistically significant.

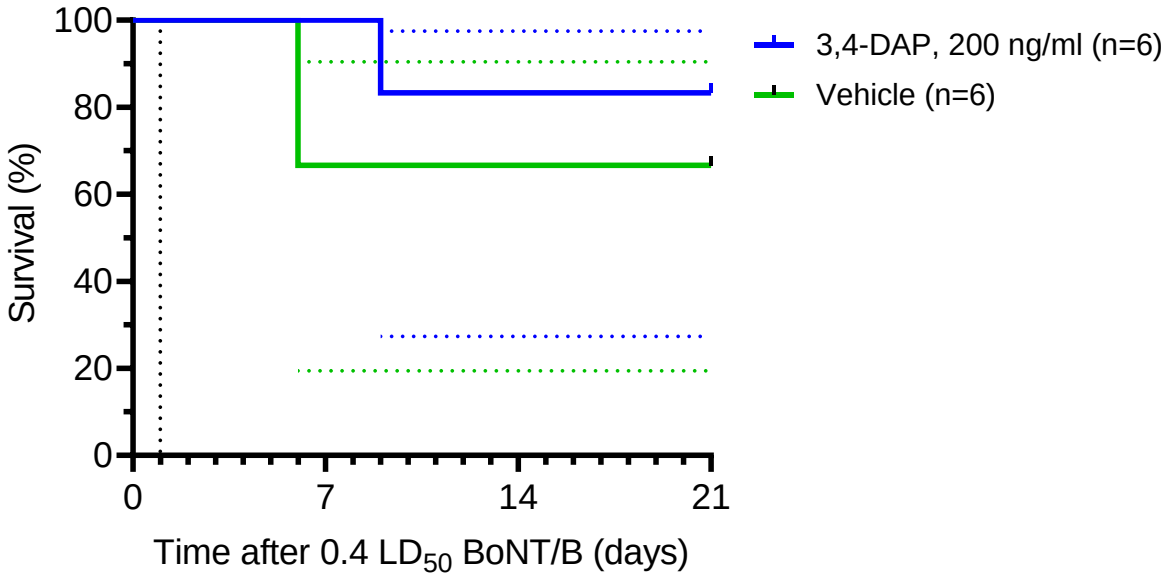

Figure 14. Survival of rats challenged with a 0.4 LD_{50} dose of BoNT/B and infused 24 hours later with vehicle or 200 ng/mL 3,4-DAP. At this dose, 2 of 6 (33%) vehicle-infused rats and 1 of 6 (17%) of 3,4-DAP-infused rats succumbed following injection of BoNT/B, which was not statistically significant. The green and blue dotted lines indicate 95% CI.

4.1.3. Weight loss. 3,4-DAP did not alter the onset nor the extent of weight loss in rats intoxicated with 0.4 LD_{50} BoNT/B (Figure 15), and body weights in both groups returned to pre-exposure levels between days 17-18.

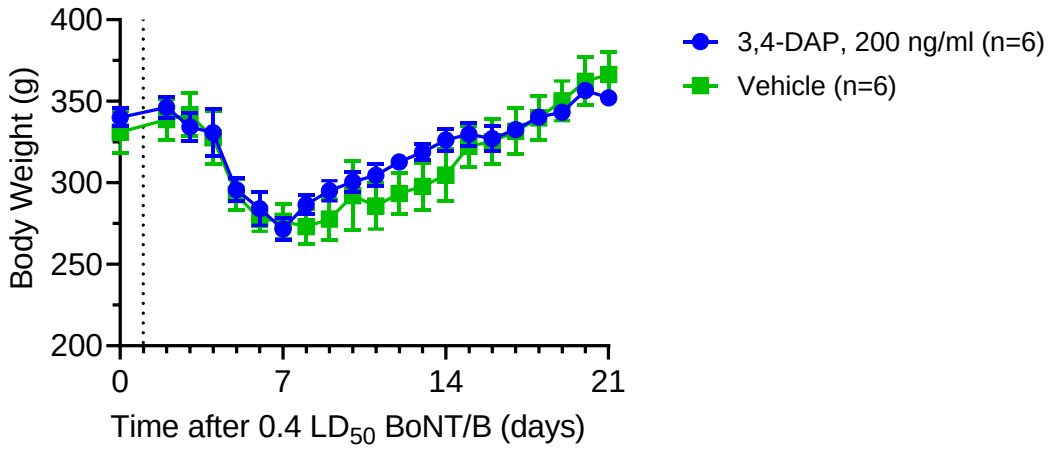

Figure 15. Body weights of rats challenged with a 0.4 LD_{50} dose of BoNT/B and infused 24 hours later with vehicle or 200 ng/mL 3,4-DAP. A similar loss of body weight was observed in both 3,4-DAP- and vehicle-infused rats.

4.2. Protection Against a 1.2 LD_{50} BoNT/B Challenge

4.2.1. CS score. The effects of 3,4-DAP (1.44 mg/kg.h, 200 ng/mL) on CS scores in rats intoxicated with a 1.2 LD_{50} challenge dose of BoNT/B are shown in Figure 16. As indicated, 3,4-DAP-infusion resulted in a moderate reduction in CS scores, with little change in onset and recovery times.

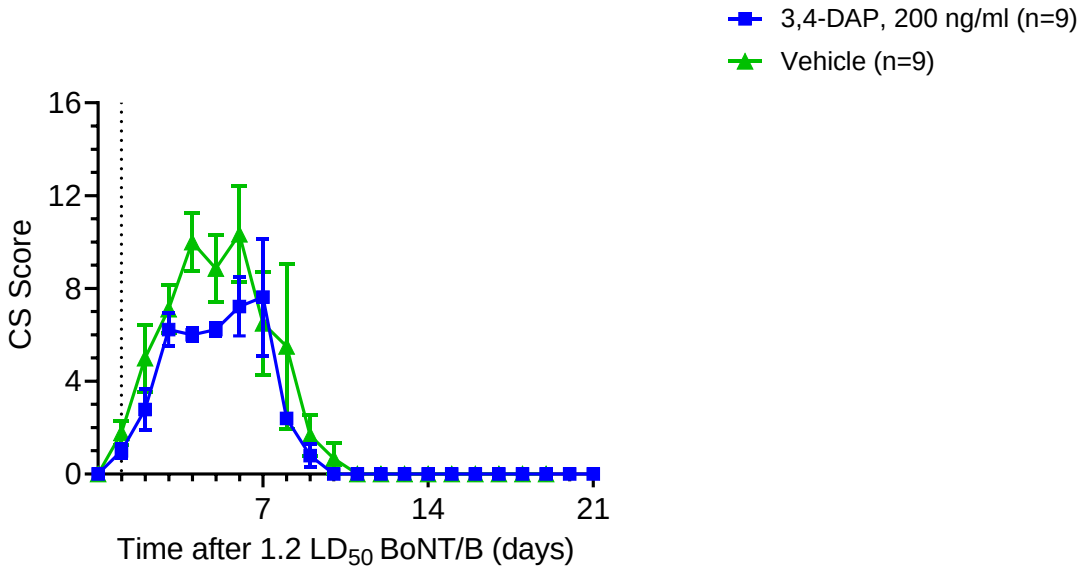

Figure 16. CS scores of rats challenged with a 1.2 LD_{50} dose of BoNT/B and infused for 14 days with vehicle or 200 ng/mL 3,4-DAP. In vehicle-infused rats, CS scores increased in severity following toxin exposure reaching a value of 10.3 on day 6. Rats infused with 3,4-DAP had less severe CS scores (maximum value = 7.6 at day 7) and slightly delayed onset and recovery times.

4.2.2. Survival. 3,4-DAP was moderately effective in protecting rats against intoxication by a 1.2 LD_{50} challenge dose of BoNT/B as indicated by increased survival in the drug-infused infused rats (Figure 17).

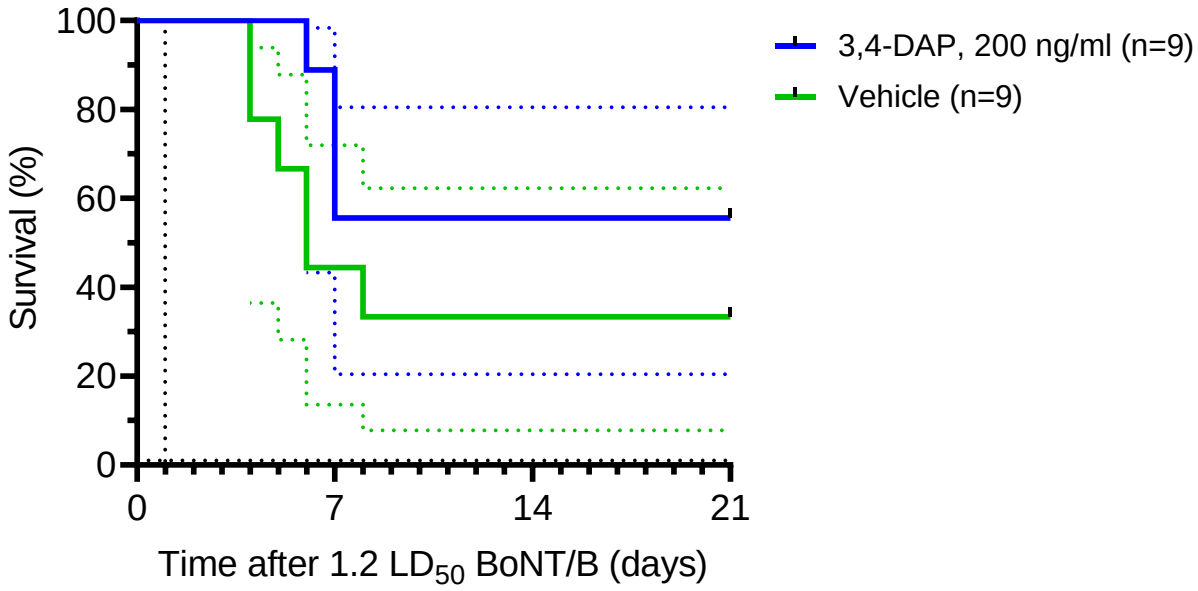

Figure 17. Survival of rats challenged with a 1.2 LD_{50} dose of BoNT/B and infused 24 hours later with vehicle or 200 ng/mL 3,4-DAP. 3,4-DAP afforded partial protection of rats from the lethal actions of 1.2 LD_{50} BoNT/B as evidenced by findings that 6/9 (67%) of vehicle-infused rats but only 4/9 (44%) of 3,4-DAP-infused rats succumbed over the 21-day period of observation; differences in survival curves between the two groups, however, were not statistically significant. Median survival was 6 days for the vehicle-infused rats and undefined for the 3,4-DAP-infused group, since lethality was below 50%. The green and blue dotted lines indicate 95% CI.

4.2.3. Body weight. 3,4-DAP had no effect on onset, extent or recovery of body weight in rats intoxicated with 1.2 LD_{50} dose of BoNT/B (Figure 18).

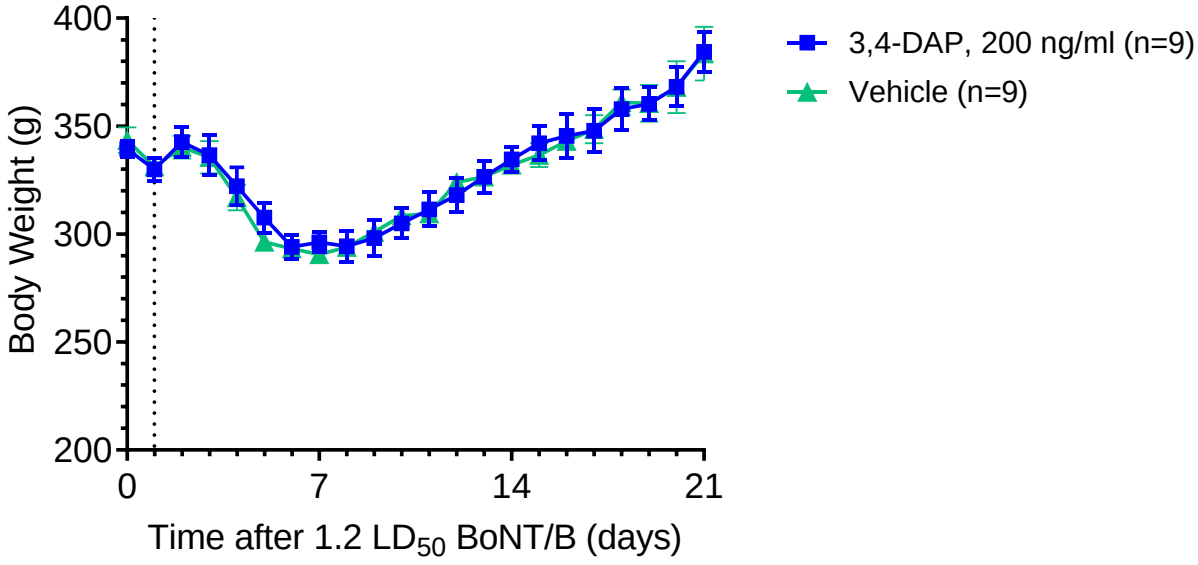

Figure 18. Body weights of rats challenged with a 1.2 LD_{50} dose of BoNT/B and infused 24 hours later with vehicle or 200 ng/mL 3,4-DAP. A similar loss of body weight was observed in both 3,4-DAP- and vehicle-infused rats. Note that in contrast with the general lack of recovery of body weight after BoNT/A intoxication at 1.2 LD_{50}, rats intoxicated with 1.2 LD_{50} BoNT/B not only recovered the weight lost during the acute phase of botulism, but also attained the weight expected for non-intoxicated animals at the end of the 21-day period of observation.

4.3. Protection Against a 1.8 LD_{50} BoNT/B challenge.

4.3.1. CS score. The effects of 3,4-DAP against a higher dose of BoNT/B (1.8 LD_{50}) are shown in Figures 19-21. At this challenge dose, more severe signs of intoxication were observed than at the lower doses in both vehicle-infused and 3,4-DAP-infused rats (Figure 19).

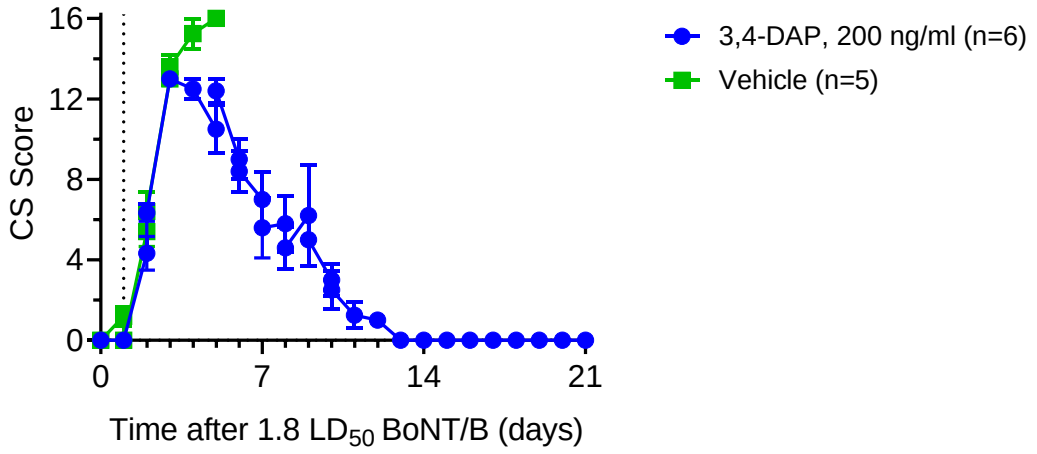

Figure 19. CS scores of rats challenged with a 1.8 LD_{50} dose of BoNT/B and infused with 1.44 mg/kg.h 3,4-DAP (200 ng/mL) for 2 weeks, beginning 1 day after challenge. Note that vehicle-infused rats exhibited signs of increasing severity for 5 days after BoNT/B challenge, culminating in mortality of all animals. CS scores of 3,4-DAP-infused rats were similar for the first 3 days but were followed by a gradual recovery over the next 10 days.

4.3.2. Survival. Between days 3 and 5, signs of intoxication had begun to decrease in the drug-infused rats but continued to increase in the vehicle-infused rats, culminating in mortality in 5 of 5 rats in the latter group. In contrast, only 2 of 6 (33%) 3,4-DAP-treated rats succumbed following exposure to a 1.8 LD_{50} dose of BoNT/B (Figure 20).

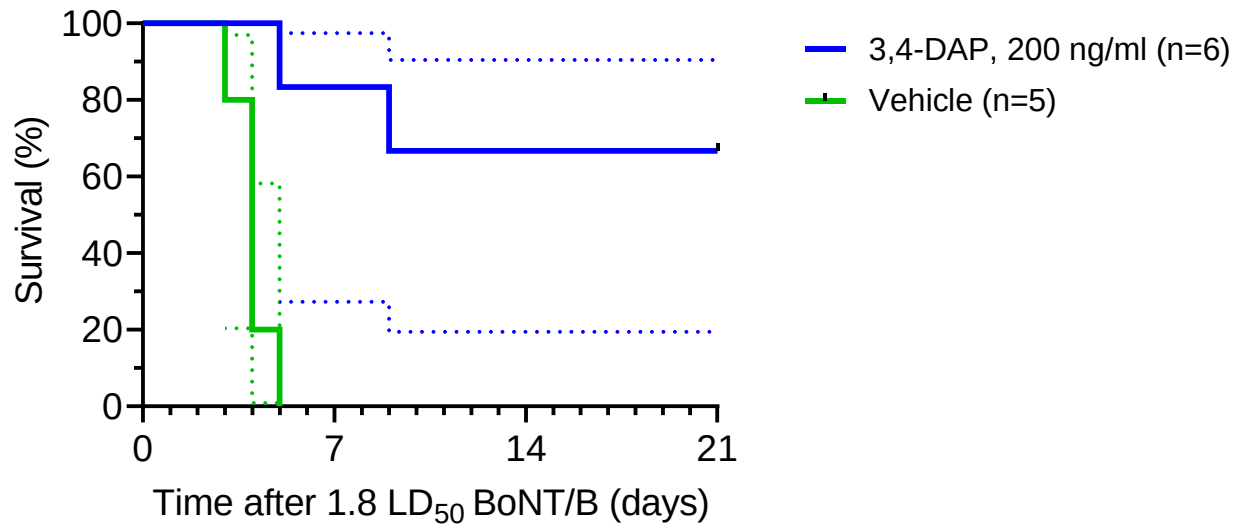

Figure 20. Survival of rats challenged with a 1.8 LD_{50} dose of BoNT/B and infused with vehicle or 200 ng/mL 3,4-DAP. All 5 of 5 vehicle-infused rats succumbed between days 3-5, whereas only 2 of 6 rats

infused with 3,4-DAP succumbed during the course of the study. This difference was highly significant by the Mantel-Cox long-rank test (P=.002). The median time to lethality in vehicle infused rats was 4 days and undefined in the 3,4-DAP infused rats since 50% mortality was not attained. The green and blue dotted lines indicate 95% CI.

4.3.3. Body weight. Since all vehicle-infused rats succumbed within 2 days of toxin exposure, it was not possible to determine whether 3,4-DAP mitigated BoNT/B-mediated loss of body weight (Figure 21). However, the body weights in surviving 3,4-DAP-treated rats did not recover to pre-exposure levels at the end of the 21 period of observation (Figure 21).

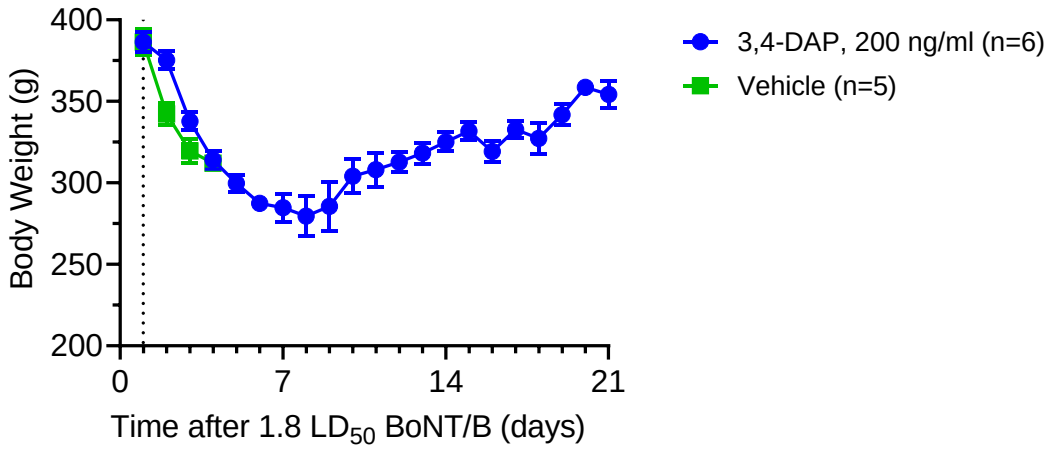

Figure 21. Body weights of rats challenged with a 1.8 LD_{50} dose of BoNT/B and infused 1 day later with vehicle or 200 ng/mL 3,4-DAP. The maximum loss in body weight in 3,4-DAP-infused rats was 27% at 8 days after challenge.

Based on the limited efficacy of 3,4-DAP against 0.4 and 1.2 LD_{50} doses of BoNT/B, the marked efficacy observed against a 1.8 LD_{50} of BoNT/B was unexpected. The data at 0.4 and 1.2 LD_{50} are more consistent with the partial efficacy observed in isolated tissues and in *in situ* nerve-muscle preparations following local injection of BoNT/B (Adler et al., 1996). Although it may be tempting to consider the results at 1.8 LD_{50} BoNT/B as an anomaly, the data do show a high level of internal consistency.

5. Protection by 3,4-DAP Against Intoxication by BoNT/E. BoNT/E is responsible for approximately 15% of foodborne botulism outbreaks in the U.S. Although BoNT/E cleaves the same substrate as BoNT/A (SNAP-25), the latter cleaves 9 residues from the C-terminus of SNAP-25, whereas the former cleaves 26 residues from the C-terminus of this SNARE protein. It has been proposed that the more extensive cleavage of SNAP-25 by BoNT/E makes the truncated SNARE protein less responsive to reversal by 3,4-DAP. This has been demonstrated in both *in vitro* and *in situ* nerve muscle preparations (Adler et al., 1995, 1996, 2000).

As with BoNT/A and BoNT/B, mice and rats were used to establish the i.v. LD_{50} of BoNT/E, and this value was used for testing the effect of 3,4-DAP against low and high challenge doses of BoNT/E in catheterized rats. Because BoNT/E-mediated toxicity has a rapid onset, infusions of 3,4-DAP were initiated 12 h after intoxication at all challenge doses.

5.1. Protection against a 0.4 LD_{50} BoNT/E Challenge

5.1.1. CS score. Following intoxication with a 0.4 LD50 dose of BoNT/E, CS signs in both vehicle- and 3,4-DAP-infused rats developed rapidly, reaching peak values at day 1.5 and recovered to baseline at day 4 (Figure 22). Both onset and recovery times were considerably faster than those observed after exposure to an equitoxic dose of BoNT/B (*cf.* Figure 13). This presents certain challenges for treatment, as will be considered in the Discussion. 3,4-DAP reduced the intensity of CS signs from 6.8 ± 1.2 to 3.4 ± 0.25.

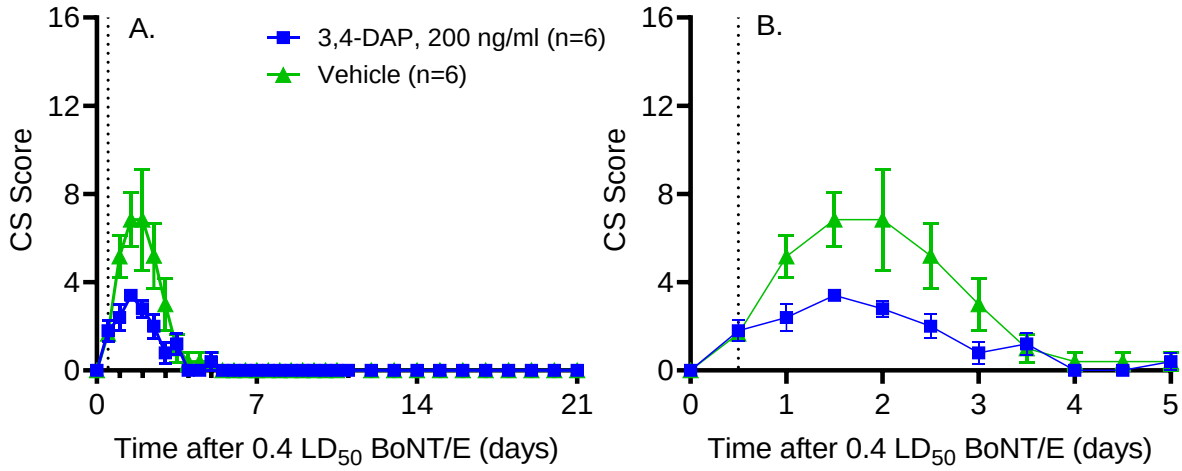

Figure 22. CS scores of rats challenged with a 0.4 LD_{50} dose of BoNT/E and infused with 200 ng/mL 3,4-DAP for 14 days, beginning 12 h after challenge. Two CS observations per day were made from day 0 to day 11 and 1 observation per day, thereafter. A. Complete time course; B. Abscissa expanded to show more detailed time course over days 0-5.

5.1.2. Survival. The effect of 3,4-DAP on survival is shown in Figure 23. In vehicle-infused rats, a 0.4 LD_{50} dose of BoNT/E led to the mortality in 1 of 6 rats (at day 3). The five-remaining vehicle-infused rats and all six 3,4-DAP-infused rats survived the 2-week infusion period plus the 1-week post-infusion period.

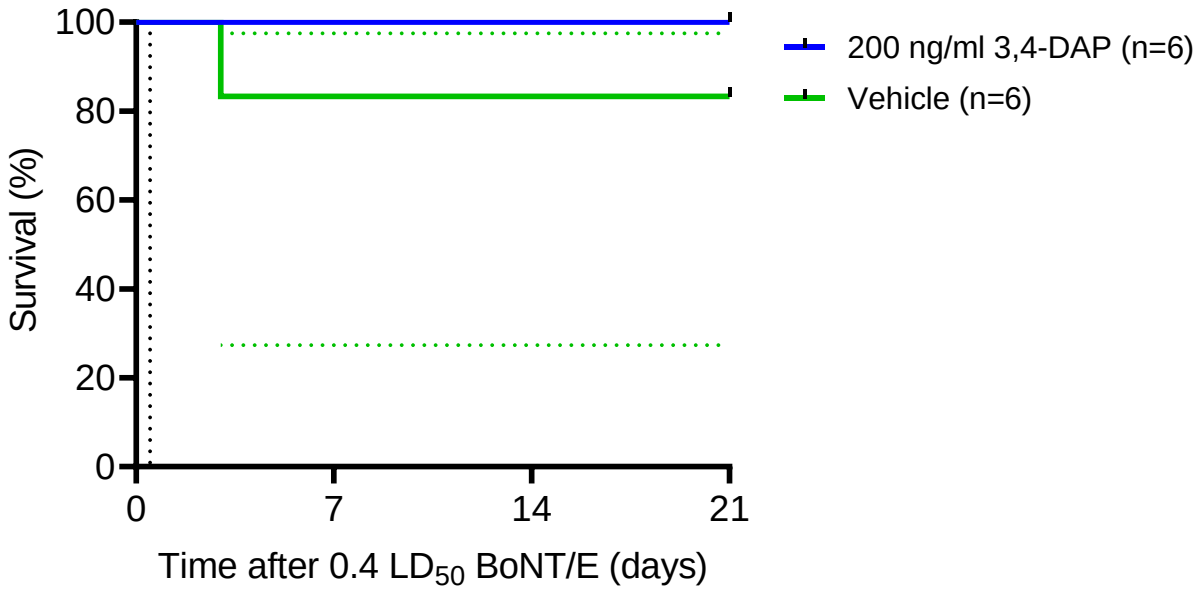

Figure 23. Survival of rats challenged with a 0.4 LD_{50} dose of BoNT/E and infused with vehicle or 200 ng/mL 3,4-DAP. One vehicle-infused rats succumbed three days after onset of infusion. No mortality was observed in the 3,4-DAP-infused group nor additional mortality in the vehicle-infused group. BoNT/E was injected at 0-time; 3,4-DAP infusion was started 12 h after intoxication as indicated by the vertical dotted line. Differences in the two survival curves were not statistically significant by the Mantel-Cox log-rank test. The horizontal dotted lines indicate 95% CI for the vehicle-infused rats.

5.1.3. Body weight. The effect of 3,4-DAP on BoNT/E-induced loss of body weight is shown Figure 24. In the vehicle-infused group, rats lost body weight for the first 2 days after BoNT/E intoxication, followed by recovery in body weight on day 6 to values slightly above pre-intoxication levels (327.2 ± 11.8 g *vs*. 322 ± 5.2 g). In the 3,4-DAP-infused group, body weights were also reduced for the first 2 days but to a lesser extent and recovered to pre-intoxication levels by day 4. Thereafter, body weights increased rapidly in both groups, although values in the 3,4-DAP group remained consistently above those in the vehicle-infused group. Such rapid recovery of body weight was not observed with BoNT/A or BoNT/B and appears to be unique to BoNT/E. This presumably stems from the relatively brief intracellular persistence of BoNT/E light chain in motor neuron terminals (Adler et al., 2001).

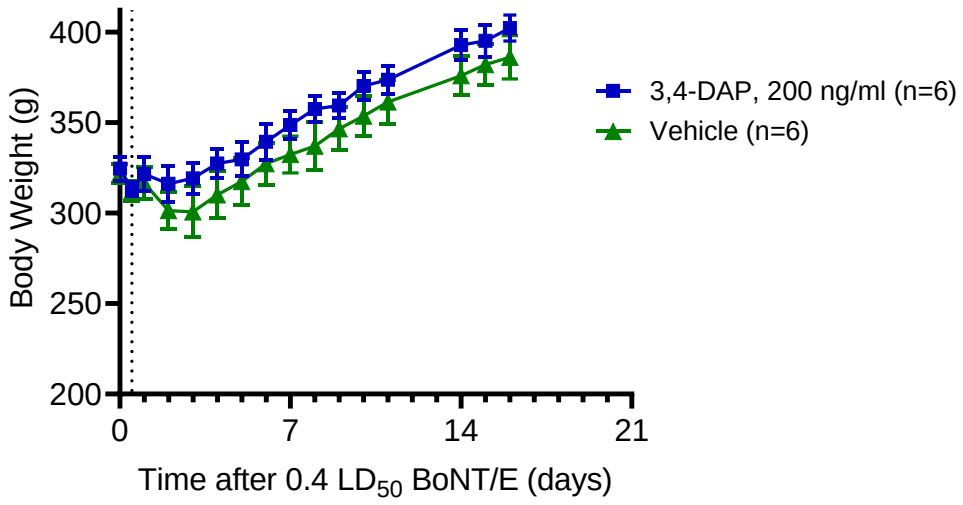

Figure 24. Body weights of rats after intoxication by BoNT/E. 3,4-DAP-infused rats had less extensive weight loss and more rapid recovery than those infused with vehicle.

5.2. Protection Against a 1.2 LD_{50} BoNT/E Challenge.

5.2.1. SC score. The effects of 3,4-DAP against a 1.2 LD_{50} challenge dose of BoNT/E on CS scores, survival and body weights are shown in Figures 25-27. In agreement with observations at the lower challenge dose (0.4 LD_{50}), rats exposed to 1.2 LD_{50} BoNT/E exhibited both a rapid onset and rapid recovery of toxic CS signs.

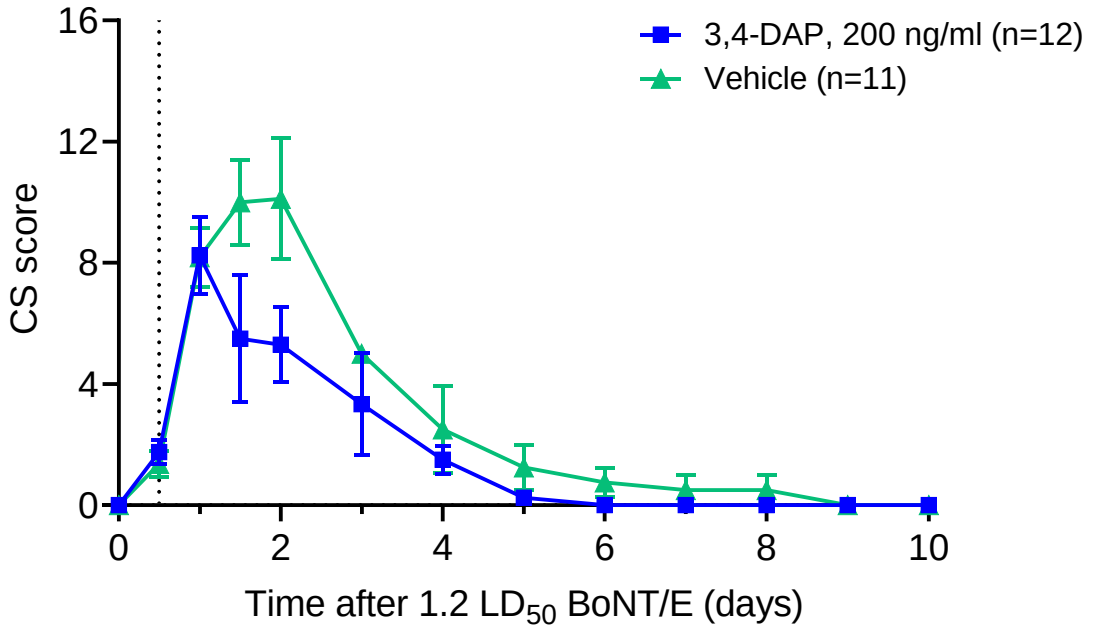

Figure 25. CS scores of rats challenged with a 1.2 LD_{50} dose of BoNT/E at 0-time and infused 12 hours later with vehicle or 200 ng/mL 3,4-DAP for 14 days (of which 10 are shown). Twelve hours coincided with first signs of intoxication (lethargy and porphyrin staining of the eyes). More severe signs such as hindlimb weakness and impaired respiration was first observed on day 2. At this BoNT/E dose, 3,4-DAP did not delay the development of toxic signs but did reduce their severity (Peak CS score = 8.2 ± 1.3 in 3,4-DAP-infused rats *vs.* 10.1 ± 2.0 in vehicle-infused rats) and accelerated the time course of recovery.

5.2.2. Survival. The median survival was 2 days in vehicle-infused rats and undefined in 3,4-DAP-infused rats, since mortality in this group did not reach 50% (Figure 26). Three of 12 rats infused with 3,4-DAP and 6 of 11 vehicle-infused rats succumbed during the 21 period of observation. Mortality occurred early in both groups: 1 and 3 days in DAP-infused rats and 1.5 and 2 days in the vehicle-infused group.

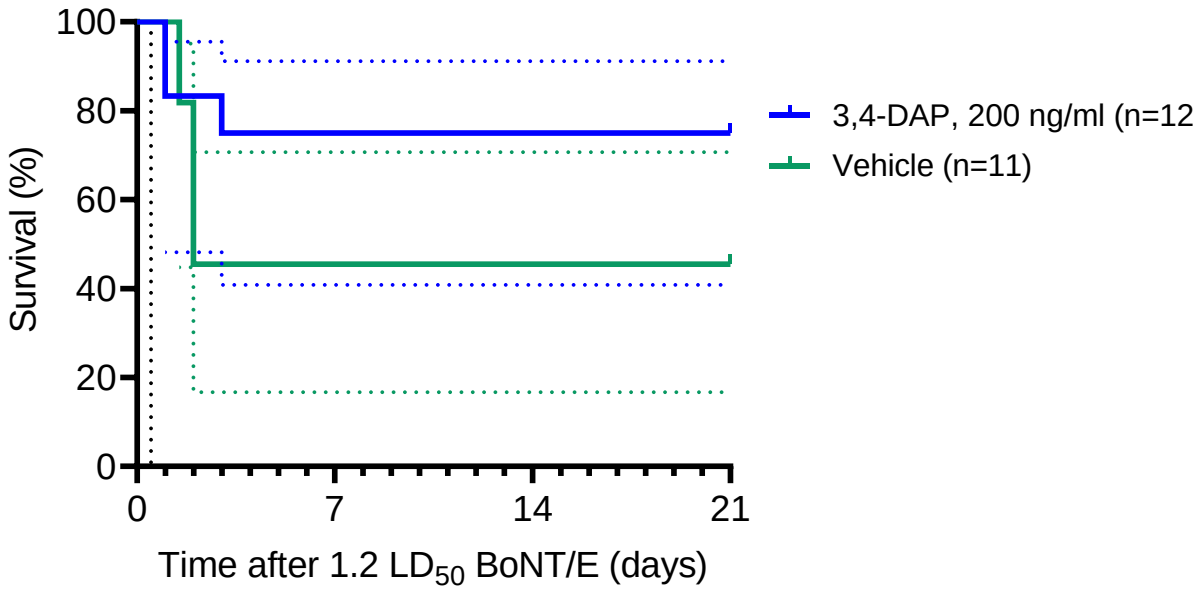

Figure 26. Survival of rats challenged with a 1.2 LD_{50} dose of BoNT/E at 0-time and infused 12 hours later (vertical dotted line) with vehicle or 200 ng/mL 3,4-DAP. The blue and green dotted lines indicate 95% CI for 3,4-DAP- and vehicle-infused rats, respectively. Differences in the two survival curves were not statistically significant by the Mantel-Cox log-rank test.

5.2.3. Body weight. In both 3,4-DAP- and vehicle-infused rats, weight loss was evident 2 days after BoNT/E injection. Weight gain was observed 1 day later in 3,4-DAP-infused rats and 3 days later in vehicle-infused rats. As with the lower dose of BoNT/E (Figure 24), vehicle-infused rats regained weight in parallel with 3,4-DAP infused rats but maintained a lower body weight during the recovery phase.

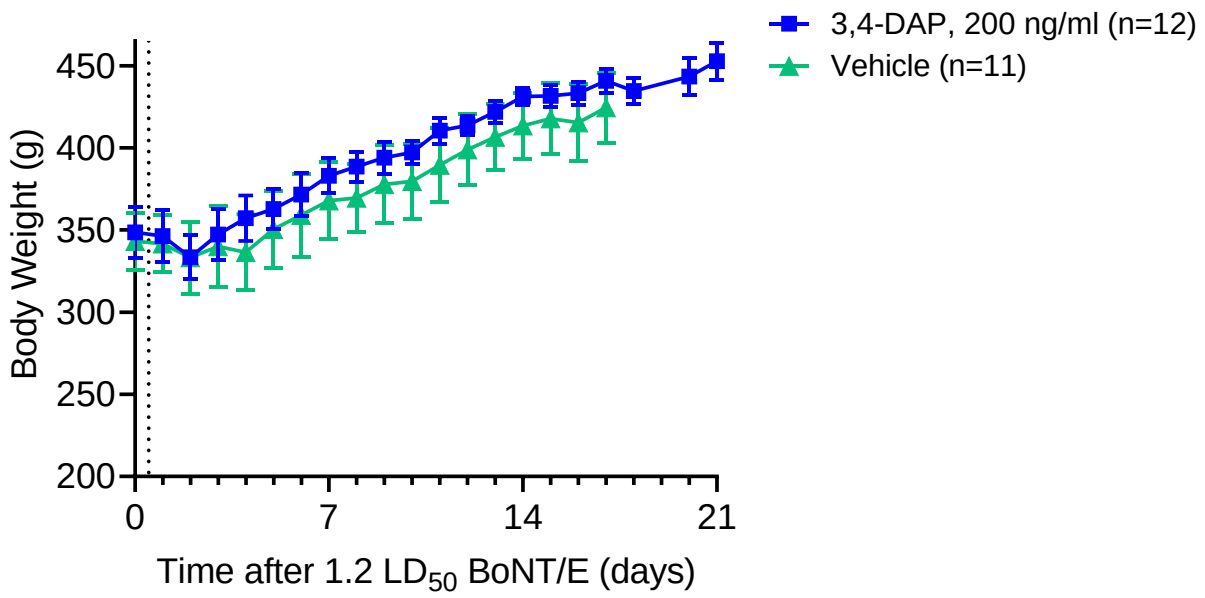

Figure 27. Body weights of rats challenged with a 1.2 LD_{50} dose of BoNT/E and infused 12 hours later with vehicle or 200 ng/mL 3,4-DAP.

6. DISCUSSION

Botulism is a rare but deadly disease with an annual incidence of 110 cases in the U.S. and a fatality rate of 5-10% (Sobel, 2005). Current treatment for botulism consists of slow i.v. infusion of equine heptavalent antitoxin (BAT®), followed by intensive/supportive care until sufficient muscle strength recovers to allow for return of spontaneous ventilation and basic motor function (O'Horo et al., 2018). However, since BoNT LC acts by cleaving SNARE proteins inside the nerve terminal and antitoxin can only neutralize BoNT in circulation, the efficacy of antitoxin decreases as intoxication progresses, since fewer circulating toxin molecules remain available to neutralize. Much of the efficacy of antitoxin is lost if it is administered >48 h after onset of intoxication or after the onset of obvious muscle weakness (Tacket et al., 1984; Fan et al., 2016). Thus, there is an urgent need for treatments that are not limited by the short time window of antitoxins, especially in light of the long duration of BoNT intoxication, which in severe cases can exceed 6 months (Keller et al., 1999; Adler et al., 2001).

6.1. Limitations of Small Molecule Inhibitors (SMIs) as Potential BoNT Countermeasures.

To address the need for post-exposure treatments, considerable effort has been focused on discovery of small-molecule LC inhibitors using conventional medicinal chemistry approaches. Thus far, however, none of the candidates have demonstrated robust *in vivo* efficacy in animal models (Duplantier et al., 2016; Jacobson et al., 2017; Adler et al., 2019), and most exhibited low efficacy even in cell-based systems (Dickerson et al., 2014). The lack of success of SMIs may be explained by the difficulties of addressing three key challenges in the design of BoNT inhibitors. First, drugs targeting the LC must be cell permeant in order to gain access to the LC after the latter is internalized in the nerve terminal cytosol. Second, the SMI would need to have exceptionally high potency, since any uninhibited catalytic activity of the LC would result in continued SNARE protein cleavage. Third, the LCs of BoNT/A and BoNT/B are highly persistent in neurons (Keller et al., 1999; Adler et al., 2001), whereas SMIs, in comparison, have exceedingly short biological half-lives. Consequently, effective LC inhibitors must be able to produce sustained and near complete inactivation of the LC. This would allow replacement of the cleaved SNARE proteins via *de novo* synthesis for restoration of neuromuscular transmission and muscle function.

6.2. Medical Countermeasures That Act by Enhancing Transmitter Release.

It has been proposed that neuromuscular paralysis can be overcome by drugs that enhance cholinergic neurotransmission such as K^+ channel blockers (Otsuka and Edno, 1960; Lundh et al., 1977; Molgó et al., 1980; 1987; Kalia and Swartz, 2011) and in more limited cases by cholinesterase inhibitors such as pyridostigmine or edrophonium (Cherington 1974; Young and Halstead, 2014). We and others have shown that the aminopyridines, notably 3,4-DAP, can transiently reverse muscle paralysis in a dose/concentration-dependent manner after intoxication by BoNT, especially for intoxication caused by serotype A (Adler et al., 1995; 1996, 2000, Bradford et al. 2018). 3,4-DAP is a K^+ channel blocker that prolongs neuronal action potential duration, thereby increasing the release of ACh, thus compensating for the inhibitory action of BoNT on transmitter release (Südhof and Rizo 2011; Bradford et al., 2018). The advantages of 3,4-DAP over other aminopyridines that have been studied (e.g., 4-aminopyridine, 4-AP) is that 3,4-DAP is 6-10-fold more potent in increasing evoked transmitter release and has fewer central nervous system off target effects (e.g., seizures, dizziness, paresthesia, balance disorders) because it is less likely than 4-AP to cross the blood-brain-barrier (Vohra et al., 1965; Molgó et al., 1980). The principal benefit of using 3,4-DAP for treatment of botulism is that it is not restricted by a narrow time window and does not require the LC to be inhibited, neutralized or removed from the nerve terminal to exert its action. In addition, although 3,4-DAP and other K^+ channel blockers are most effective against BoNT/A, they are also effective, albeit to a lesser degree, against BoNT/B and BoNT/E intoxication.

6.3. Clinical Studies with K^+ Channel Blockers.

It has been known since the last century that guanidine (a derivative of the nucleic acid guanine) and some of its congeners have the ability to enhance the sensitivity of skeletal muscle to ACh (Frank et al., 1923). This finding led investigators to test the efficacy of guanidine in myasthenic patients, even though the mechanism of action of guanidine (K^+ channel blockade) and the underlying cause of myasthenia gravis (autoimmune disease targeting nicotinic ACh receptors) were not yet known (Minot et al. 1938). The authors noted that guanidine caused a rapid but transient improvement in muscle tension, and myasthenic patients treated with guanidine reported subjective feelings of increased strength and well-being. Since the muscle weakness associated with neuromuscular diseases such as myasthenia gravis and especially the Lambert-Eaton variant (LEMS) shares similarities with botulism (Ricker et al. 1971), the K^+ channel blockers guanidine, 4-AP and 3,4-DAP were tested in selected botulinum intoxicated patients beginning in 1968 (Cherington and Ryan, 1968; Kaplan et al., 1979; Friggeri et al., 2013). In the majority of cases, ptosis, extraocular palsies and strength in the extremities improved while the drugs were in circulation, but symptoms reappeared following metabolic clearance of the drugs.

In all reported cases, patients treated with K^+ channel blockers still required artificial ventilation, until sufficient toxin was eliminated from motor nerve terminals to allow for return of spontaneous ventilation (Cherington and Ryan, 1968, 1970). Cherington (1974) speculated that the respiratory muscles may be more sensitive to BoNT than limb muscles or perhaps less sensitive to the actions of K^+ channel blockers to explain the resistance of respiratory muscle to K^+ channel blockers (Cherington, 1974; Puggiari and Cherington, 1978). Neither, however, is likely since diaphragm muscles (the principal muscles of respiration) have not been found to exhibit higher sensitivity to BoNT than limb muscles and showed equal sensitivity to the actions of K^+ channel blockers (Adler et al., 1995, 2012). A more likely possibility is that nerve terminals that innervate diaphragm muscles take up toxin at a greater rate than those innervating limb muscles because the former have a higher duty cycle, a key determinant of toxin uptake (Rogozhin et al., 2008).

While the results from human case studies are encouraging, they fall short of being able to provide a clear indication of the role K^+ channel blockers would have in the treatment of botulism. Because of the low incidence and sporadic nature of botulism outbreaks, and the fact that none of the K^+ channel blockers were FDA-approved at the time the human case studies were performed, it was not possible to design adequately powered clinical trials. The studies described above lacked consistency in factors such severity of intoxication, dosage of drug, duration of treatment, route of administration, time from onset of intoxication to drug administration and importantly, whether patients also received antitoxin treatment.

The phosphate salt of 3,4-DAP was approved by the FDA in 2018 for treatment of LEMS under the proprietary name Firdapse®. LEMS is a rare autoimmune disease in which antibodies target and destroy Ca^{2+} channels at motor nerve terminals, leading to inhibition of ACh release (Zhang et al., 2021). LEMS is characterized by muscle weakness and autonomic dysfunction and resembles moderate BoNT intoxication in signs and symptoms. The well-controlled clinical trials that preceded approval of Firdapse® can provide a valuable database for the safety, efficacy and pharmacokinetic profile of this drug in humans that can be used for rational selection of doses and other parameters for future studies in botulinum intoxicated patients.

In addition, based on our data with continuous infusion, it would be important to perform studies in humans using long-term infusion for drug delivery. Infusion times would need to be adjusted for the different durations of BoNT intoxication, which vary with serotype and severity of intoxication (Keller et al., 1999; Adler et al., 2001). In humans, recovery from BoNT/A intoxication can take as long as 6 months, whereas recovery from BoNT/E may only take 3-4 weeks and recovery from BoNT/B would generally fall between these 2 values (Cherington, 1998; Marcus 2006).

6.4. Salient Findings in the Current Study.

6.4.1. Efficacy of 3,4-DAP against BoNT/A.

The effects of 3,4-DAP infusion were tested at 1, 2.5 and 4 LD_{50} doses of BoNT/A. 3,4-DAP afforded complete protection at 1 and 2.5 LD_{50} doses of BoNT/A (Figures 2 & 5). In the absence of 3,4-DAP, 3 of 6 rats at 1 LD_{50} and 6 of 6 rats at 2.5 LD_{50} succumbed within 6 days of challenge.

Two doses of 3,4-DAP were used for examining the efficacy of 3,4-DAP at 4 LD_{50} BoNT/A: 125 ng/mL (Figure 8) and 200 ng/mL (Figure 11). At the lower dose, 3,4-DAP increased the median time-to-death from 1 to 3.6 days, however, none of the 3,4-DAP-infused rats survived beyond day 7. At the higher infusion dose, 3,4-DAP increased the median time-to-death to 10 days and 1 of 6 (17%) 3,4-DAP-infused rats survived the entire 14-day infusion time plus the 7-day post infusion period. We did not explore higher 3,4-DAP infusion doses, since 200 ng/mL is at the upper end of the dose used for treatment of LEMS (Thakkar et al., 2017). In principle, higher doses of 3,4-DAP would likely have conferred more robust protection at 4 $LD_{50,}$ since in isolated diaphragm preparations, maximal protection was achieved using 100 µM 3,4-DAP, a concentration that would be lethal *in vivo* (Adler et al., 2012). By comparison, 200 ng/mL is equivalent to 1.83 µM.

6.4.2. Efficacy of 3,4-DAP against BoNT/B.

Since 3,4-DAP is known to be less effective against BoNT/B and BoNT/E (Adler et al., 1996), studies with these serotypes were performed with lower doses of toxin. At a BoNT/B or BoNT/E dose of 0.4 LD_{50}, 3,4-DAP reduced the severity of CS scores (Figures 13 &22) and shortened the time course of recovery. Since very few animals succumbed to botulism at this challenge dose, it was not possible to determine whether 3,4-DAP protected rats from lethality (Figures 14 & 23).

At the challenge dose of 1.2 $LD_{50,}$ 3,4-DAP was still effective in lowering overall CS scores, but the acceleration of recovery was less apparent in BoNT/B-intoxicated rats (Figure 16) than in rats intoxicated with BoNT/E (Figure 25). Mortality was reduced by 3,4-DAP for both serotypes from 67% to 44% for BoNT/B (Figure 17) and from 55% to 25% for BoNT/E (Figure 26); however, these changes were not statistically significant. For BoNT/B, a higher dose of 1.8 LD_{50} was also examined. At this dose, all of the vehicle-infused rats succumbed to botulism between days 3-5, whereas 4 of 6 (67%) of 3,4-DAP-infused rats survived the full 21 days of the study (Figure 20). This was an encouraging, albeit unexpected, finding based on the results at 0.4 and 1.2 LD_{50} doses of BoNT/B. It would be of considerable interest to reexamine this dose in a future study since confirmation of this finding would make 3,4-DAP a more attractive candidate for development as a medical countermeasure for BoNT intoxication.

In an earlier study, Siegel et al. (1986) examined the efficacy of high doses of 3,4-DAP (4-8 mg/kg) against 10, 20 and 40 LD_{50} doses of BoNT/A, BoNT/B or BoNT/E. 3,4-DAP was injected 3 h after intoxication and hourly thereafter for up to 32 h, which was the end of their study. Surprisingly, 3,4-DAP was effective against BoNT/A even at these high doses, increasing the median time-to-death and protecting over 50% of mice from lethality. No protection or significant increase in time-to-death was observed with any of the other serotypes under the same experimental conditions. Based on the above it would be of interest to explore higher 3,4-DAP infusion doses and higher challenge doses of BoNT to determine the limit of protection with 3,4-DAP. Rodents appear to tolerate 3,4-DAP plasma levels of 1000 ng/mL (Ishida et al. 2019), which is 5 times greater than the high dose used in the present study.

7. CONCLUSION

These data demonstrated a potential treatment for BoNT/A by the free base formulation of an FDA approved drug. Collectively, they present a compelling argument for continuing studies aimed at repurposing Firdapse® (3,4-DAP phosphate) for treatment of natural outbreaks of botulism, iatrogenic botulism as well as for treatment of botulism that may result from a bioterrorist attack.

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PART 2 - Determine LD50 of BoNT/A in the Rabbit

1. INTRODUCTION AND RATIONALE

The preceding studies with demonstrated that the FDA approved drug 3,4-diaminopyridine (3,4-DAP) was able to protect rats against BoNT/A-induced toxicity and lethality at challenge doses up to 4 LD_{50}. 3,4-DAP also showed some promise for alleviating the toxic signs of exposure to BoNT/B and BoNT/E. These 3 serotypes account for most of the outbreaks of botulism worldwide.

To perform more advance studies, it is necessary to develop a large animal model to be able to carry out pharmacokinetic and other studies with 3,4-DAP. Non-human primates (NHPs) were traditionally used for carrying out such studies for BoNT therapeutics (e.g., heptavalent antitoxin) under the FDA's animal rule. NHPs, however, have become difficult to procure and are cost prohibitive. New Zealand rabbits have been proposed as a replacement for NHPs and have the following attributes: they are sensitive to all 7 BoNT serotypes and studies by a colleague at the Israel Institute of Biological Research have shown that they can be kept alive at near lethal doses of BoNT for at least one month with supportive care (Torgeman et al., 2021). Moreover, the onset and progression of intoxication can be monitored quantitatively by measurement of respiratory minute volume, thus providing a trigger-to-treat model. Finally, Torgeman et al. (2021) demonstrated that rabbits can be infused with drugs such as 3,4-DAP using a wearable infusion pump. The study described in this report was designed to determine the LD_{50} of BoNT/A in the rabbit, as a first step to studies of evaluating the efficacy of 3.4-DAP in rabbits challenged with BoNT/A, BoNT/B and BoNT/E.

2. METHODS AND GENERAL APPROACH

2.1. Preparation of toxin stock solution.

A new vial of BoNT/A (Lot no. A113021-01) was purchased from Metabiologics, Inc. (Madison, WI) at the start of the project. The vial contained 100 µg of pure BoNT/A neurotoxin in a 100 µL aqueous solution containing 0.2% gelatin, 100 mM sodium phosphate and 50 mM sodium chloride (pH 7.4), with a nominal specific activity of 2.5 x 108 mouse LD50 units (MU) per mL. To prepare stock solutions, the vial was thawed and a 5 µL aliquot was removed and diluted with 495 µL of sterile phosphate buffered saline (PBS) containing 0.2% gelatin (gelatin phosphate buffer, GPB) to yield a 10 ng/µL toxin solution. This stock solution was dispensed in 20 µL aliquots and frozen at -80 oC until use. Prior to each experiment, one tube of the stock solution was further diluted with GPB solution as appropriate to obtain working concentrations suitable for toxicity testing. The role of gelatin was to preserve stability of toxin during successive dilutions. A new vial of the 10 ng/µL stock solution was used for each experiment to minimize potential loss of BoNT/A activity from freeze-thaw damage. All toxicity studies described in this report were performed with dilutions of the original 5 µL aliquot of BoNT/A, and each vial of toxin was subjected to the same number of freeze-thaw cycles (2) to ensure consistency. Toxin dilutions were carried out in a class II, type A2 biosafety cabinet.

2.2. Supportive care for BoNT/A-intoxicated rabbits.

Supportive care is required to prevent BoNT/A-intoxicated rabbits from dying due to dehydration or starvation and is appropriate since similarly intoxicated humans would receive supportive care in a hospital setting (Rao et al., 2021). Thus, rabbits that were unable to reach food sources due to muscle weakness and limited mobility were fed nutritional gels such as DietGel Boost®. More severely intoxicated rabbits also received subcutaneous (s.c.) injections of an electrolyte solution such as Duphalyte® diluted 1:1 with 0.9 saline (50 mL/injection) twice daily to treat dehydration.

2.3. Determine the intraperitoneal (i.p.) LD_{50} of BoNT/A in mice to establish the specific activity of BoNT/A lot no. A113021-01.

Because of the steep dose response curve for BoNT/A-mediated toxicity, small lot-to-lot variations in specific activity can dramatically affect lethality (Sesardic et al. 2003). Potency testing must therefore be carried out by the user when precise knowledge of specific activity is essential. In accordance with convention, we determined the potency of BoNT/A with the mouse lethality assay using i.p. administered toxin as described by Pearce et al. (1994). Five doses of BoNT/A were tested with 10 mice per dose. The doses were selected to approximate the Metabiologic's nominal LD_{50} of 0.20 ng/kg and were incremented or decremented by factors of ~1.5 as suggested by Pearce et al. (1994).

In the dose range studied (0.096 - 0.480 ng/kg), the first sign of intoxication was generally the presence of a "wasp waist," which is an indication of diaphragmatic weakness. This was observed across all doses and was usually detectible within 1 day of exposure. With time, additional signs were observed, most frequently lethargy often accompanied by hind-limb weakness. The majority of mice in the higher dose groups (≥ 0.216 ng/kg) exhibited these additional signs on day 1 and progressed to more extensive paralysis, culminating in respiratory distress and mortality by day 2 or 3 after exposure (Table 1). By day 3, 20 of 30 mice (67%) at

the 3 highest doses were found dead or met criteria to be humanely euthanized. Mice were euthanized when they exhibited respiratory distress, gross hematuria, excessive weight loss (> 35%), or attained cumulative a Clinical Severity (CS) score of 16 or greater. Euthanized animals were counted the same as those that were found dead. Most of the deaths occurred by day 3, and only two additional deaths were observed between day 3 and day 5 (Table 1).

Table 1: Lethality of BoNT/A in CD1 Mice[a]

BoNT/A ng/kg	N=	% dead				
		Day 1	Day 2	Day 3	Day 4	Day 5
0.096	10	10	10	10	30	30
0.144	10	20	20	40	40	40
0.216	10	10	40	60	60	60
0.336	10	30	60	60	60	60
0.480	10	40	70	80	80	80

[a]Mice of either sex (21-34 g) were injected i.p. with 200 µL BoNT/A on Day 0 and observed for toxicity and lethality for a consecutive 5-day period.

The effect of BoNT/A on lethality at day 5 is plotted in Figure 1. The marked steepness of the dose-lethality relationship is apparent from the fact that lethality occurred over a narrow dose range of just over a half log unit. The LD_{50} determined from the data in Figure 1 was 0.187 ng/kg with 95% confidence interval = 0.154 - 0.225 ng/kg (GraphPad Prism, ver. 9.4.1). The value of 0.187 ng/kg corresponds to 1 mouse unit (MU)/kg for the BoNT/A preparation used in this study. A standard MU is defined for a 20 g reference mouse and is obtained by multiplying the MU/kg determined in Figure 1 by 0.020 kg, which yields a value for MU of 0.00374 ng (3.74 pg) for the toxin actually used in this study. This value is somewhat lower than the nominal mouse LD_{50} of 4.0 pg provided by Metabiologics. The MU allows comparisons of data obtained with BoNT/A preparations of different purity and specific activity and is considered to be the "gold standard" for determination of toxin potency.

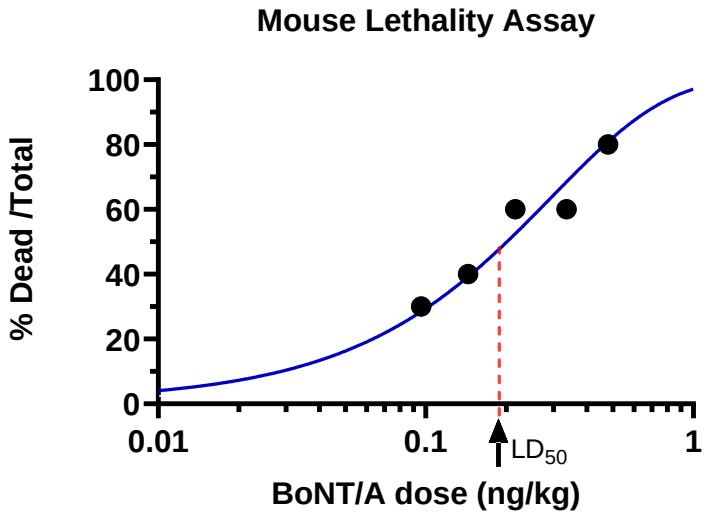

Figure 1. Mouse lethality assay. Each point represents data from 10 mice injected intraperitoneally (i.p.) with the indicated dose of BoNT/A. Signs, symptoms, and mortality were assessed twice per day. The points represent cumulative lethality at the end of 5-days of observation. The LD_{50} of our toxin preparation was determined from the variable slope-least squares fit to be 0.187 ng/kg, yielding a value of 3.74 pg for the MU (GraphPad Prism ver. 9.4.1).

2.4. Determine the intramuscular (i.m.) LD_{50} of BoNT/A in New Zealand White rabbits.

The LD_{50} of BoNT/A was determined in rabbits by the intramuscular (i.m.) route using the up-and-down procedure (UDP) of Dixon and Massey (1983). The initial weight of the rabbits at the time of dosing ranged from 2.45 kg to 2.86 kg (mean ± SEM = 2.70 ± 0.04 kg). The starting dose was based on the rabbit i.m. LD_{50} of the BoNT/A toxin complex reported by Torgeman et al. (2021) and the MU contained in our pure BoNT/A neurotoxin determined in Section 2.2. The rabbits were observed at least 3 times per day for 5 days to assess the signs and symptoms after intoxication with BoNT/A (Table 2). The stopping point for the UDP method was obtaining at least 4 live-dead reversals, which was achieved with a total of eight rabbits. Four reversals are generally used with this method, although a greater number of reversals can provide a better estimate of the LD_{50} (Zhang et al., 2022).

For the current study, the UDP method was carried out using a custom Excel program that implemented the mathematical equations of Dixon and Massey (1983); the program was validated by a biostatistician. The user input consisted of entering a starting value plus the log interval for subsequent dose progressions and then monitoring the animal for survival or mortality at the end of a specified time interval (5 days in the current study). After determining the live/dead status of the first rabbit, the program provided the next dose which was tested on the second animal; this cycle was repeated until the selected number of reversals were met (or exceeded).

3. RESULTS

3.1. LD50 of BoNT/A in New Zealand White Rabbit.

Data from the 8 rabbits used in this study are shown in Figure 2. The starting dose of BoNT/A was 0.480 ng/kg, which led an early presentation of signs (lethargy and anxiety), a rapid increase in severity (profound muscle weakness) and the animal succumbing within 2 days of exposure. This suggested that the initial dose was substantially higher than the actual rabbit i.m. LD_{50}. The next dose tested was 0.288 ng/kg, which produced a less rapid onset of signs and led to mortality within 3 days of challenge. The third dose, 0.096 ng/kg, generated only mild signs and satisfied the first reversal, since the rabbit survived the 5-day observation period (Figure 2). The initial 3 doses greatly narrowed the dose range for calculating the LD_{50}. The 5 subsequent doses were either slightly above or slightly below the anticipated lethal dose. The 8 rabbits in this study provided 5 live/dead reversals (1 more than required) and enabled the program to calculate the LD_{50} for BoNT/A as 0.141 ng/kg (95% confidence interval = 0.148 - 0.134 ng/kg). Lethality values in ng/kg can be expressed as MU/kg by dividing the LD_{50} and confidence interval by the MU of 0.00374 ng, which was derived from the mouse lethality assay (section 2.2). Converting to mouse units yields an i.m. LD_{50} for rabbits of 37.7 MU/kg (0.141 ng/kg divided by 0.00374 ng/MU), with 95% confidence intervals of 35.8 MU and 39.6 MU.

3.2. Comparison of rabbit LD_{50} with NHP LD_{50}.

The rabbit LD_{50} obtained in this study was close to the i.m. LD_{50} for BoNT/A reported in cynomolgus monkeys (*Macaca fascicularis*) of 39 MU/kg by Scott and Suzuki (1988). A notable difference, however, was that in the latter, no systemic toxicity was observed at doses <33 MU/kg, whereas in the rabbit, toxicity was observed at all doses studied. This suggests that the dose-lethality relationship in *Macaca fascicularis* may be even steeper than it is in rabbits. The i.m. BoNT/A LD_{50} in rabbits is also close to value of 40 MU/kg reported by Herrero et al. (1967) in rhesus monkeys (*Macaca mulatta*) exposed to BoNT/A by intravenous (i.v.) injection. Although the different routes of toxin administration would normally preclude comparison, this is not the case for BoNT which exhibits similar potency by i.v. and i.m. routes (Sharma, 2010).

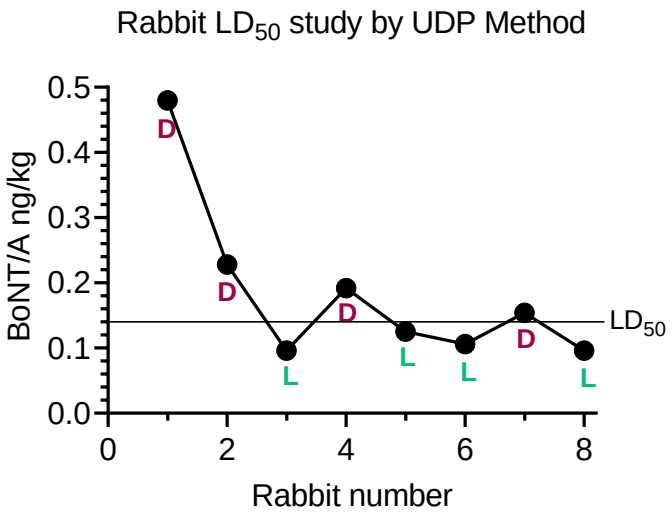

Figure 2. Rabbit LD_{50} study. Each symbol represents one rabbit exposed to the indicated dose of BoNT/A. **D** indicates that the rabbit succumbed within the 5-day period of observation; **L** indicates that the rabbit survived for 5 days after toxin exposure. Three of the 4 deaths were by euthanasia. A similar scoring system for BoNT/A-mediated toxicity was established for rabbits as that used for rats (see below). The criterion for euthanasia was attaining a cumulative score of 16 on these signs or the appearance of one or more of the following: acute respiratory distress, loss of >35% body weight, an SpO2 value of ≤ 80 (measured by pulse oximetry) and cardiac arrhythmia (determined by stethoscopic examination). Rabbits meeting the criteria for euthanasia were first injected i.m. with a combination of ketamine and dexmedetomidine for sedation and then injected i.v. with 200 mg/kg sodium pentobarbital followed by bilateral thoracotomy.

4. DISCUSSION AND CONCLUSIONS

The characteristic signs of BoNT/A intoxication in rabbits in general order of onset consisted of anxiety, lethargy, weight loss, appearance of caecotrophs (sign of impaired GI system), limb weakness (most frequently involving the hind-limb) and general paralysis (Table 2). At doses of 41.2 MU/kg (0.154 ng/kg) and 51.3 MU/kg (0.192 ng/kg), toxic signs progressed to respiratory distress (indicated by SPO_2 readings of ~80% and arrhythmia). Heart rate and SPO_2 were not determined in the first two rabbits, but a reduced frequency of respiration was observed in both animals.

In general, the onset of signs and their severity were dose-dependent; the most frequent initial signs, regardless of dose, were anxiety and lethargy. These were usually observed prior to the development of overt muscle weakness. Weight loss, which was also frequently observed, may have resulted from difficulty in swallowing, decreases in intestinal smooth muscle tone and impairment of autonomic control of the GI tract (Adler et al., 2019). The prominence of GI signs in BoNT/A intoxication may be somewhat unique to rabbits, perhaps as a consequence of their complex digestive system (Davies, 2003).

An encouraging result from this study was the finding that one can elicit clear signs of systemic botulism in the rabbit over a relatively wide dose range with animals surviving for at least 5 days. This would allow for the testing of MCMs such as protease inhibitors and aminopyridines, since *in vivo* efficacy should be demonstrable in this time frame. This study, and that of Torgeman and colleagues (2021) suggest that the rabbit is a suitable large animal model for development of MCMs for BoNT/A intoxication and can potentially fill the void created by the current shortage of typical NHP models.

Table 2. Summary of toxic signs of rabbits intoxicated by BoNT/A.

Animal No.	Dose (MU/kg)	Dose (ng/kg)	Onset of toxicity	Signs*	Comments	Time of Death/ Euthanasia**
1	128.3	0.48	3 h	1,2,3,4,5,6	Unusually rapid onset with highly severe signs	2 days***
2	77	0.288	2 days	1,2,3,4,5,6	Rapid progression from first signs (lethargy) to death	3 days
3	25.7	0.096	4 days	1,4	Mild transient signs	Survived
4	51.3	0.192	2 days	1,2,3,4,5,6	Rapid progression from first signs to death; Low SpO2 (79%); arrhythmia	2.3 days
5	33.4	0.125	3 days	1,2,3,4,6	Delayed onset but moderately severe signs from days 3 to 5	Survived
6	28.3	0.106	3 days	1,2,4,6	Major sign was weight loss; lethargy developed on day 5	Survived
7	41.2	0.154	2 days	2,3,4,6	Signs developed gradually but at day 4 arrhythmia and low SpO2 (78%) were observed	4 days
8	25.7	0.096	3 days	2,3,4,6	Delayed onset. Mild signs up to day 5	Survived

*Signs: 1 = Anxiety; 2 = Lethargy; 3 = Limb weakness; 4 = Weight loss, 5 = General paralysis, 6 = Visible caecotrophs (sign of impaired GI system).
**Rabbits were humanely euthanized if they lost >35% body weight, exhibited cardiac arrythmia or were in respiratory distress.
***days following exposure; exposure is defined as day 0.

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