Augmentation of the catalytic activity of Botulinum Neurotoxin Type B does not result in increased potency in physiological systems

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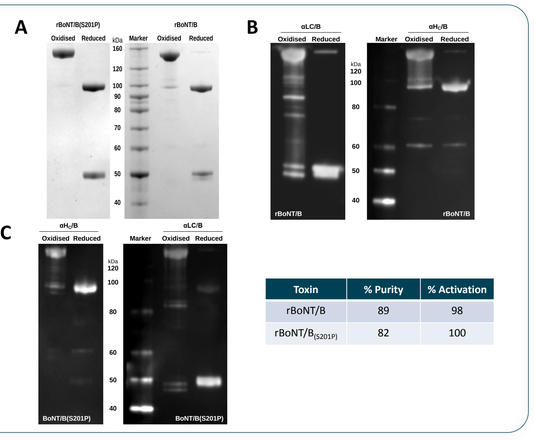
Introduction

- Botulinum neurotoxins (BoNTs) are extensively used as therapeutic agents in the clinic.
- In the treatment of muscular disorders, the clinically effective dose of BoNT/B is 40-50 times higher than that of BoNT/A when compared in equivalent mouse lethality units[1].
- Treatment with BoNT/B is associated with a higher incidence of immune response compared to BoNT/A, most likely due to the increased protein load [2].
- Lowering the required treatment dose of BoNT/B may help to lower immune response in patients.
- One way to achieve higher potency could be through mutations in the light chain (LC) of BoNT/B to enhance its catalytic activity.
- LC/B derivatives with elevated catalytic activity for VAMP-2 substrate cleavage have been described, including the mutation S201P.
- The S201P mutation increased the K_{cat} (substrate turnover) of LC/B approximately 10-fold, while the K_m (substrate affinity) was found to be unaltered[3].
- We have manufactured a full-length BoNT/B toxin incorporating the S201P mutation recombinantly in *E. coli* (rBoNT/$B_{(S201P)}$). Here, we describe the characterisation of rBoNT/$B_{(S201P)}$ in a range of *in vitro*, *ex vivo* and *in vivo* assays.

Methods & Results

Cloning and expression of neurotoxins

- The gene sequence encoding the botulinum neurotoxin serotype B1 (BoNT/B1; Okra strain) was subcloned into pET32a. The S201P mutation was introduced by site directed mutagenesis.
- BoNTs were expressed in E. coli. strain BLR DE3 and lysed by ultrasonication. Following clarification by centrifugation, the lysate was passed through an anionic exchange column.
- Target molecules were captured using a hydrophobic interaction resin (butyl Sepharose, GE) and purified using an anion-exchange resin (Q Sepharose, GE).
- The partially purified molecule was proteolytically cleaved with the endoproteinase Lys-C to yield the active di-chain, before final polishing down a second hydrophobic interaction resin (phenyl Sepharose, GE).
- Each purified molecule was desalted into PBS pH 7.2 and stored with 1 mg/mL BSA at -80 °C.

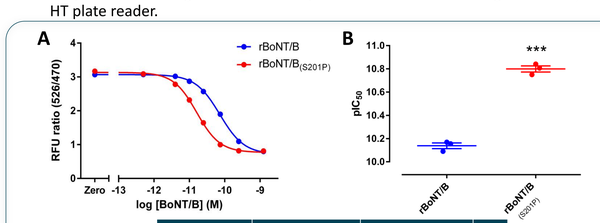


Toxin	% Purity	% Activation
rBoNT/B	89	98
rBoNT/$B_{(S201P)}$	82	100

Figure 1. A) rBoNT/B (*right*) and single mutant, rBoNT/$B_{(S201P)}$ (*left*) were purified using a four step process – anionic exchange chromatography (AEC), capture by hydrophobic interaction chromatography (HIC), purification by AEC and a final HIC polish step after proteolytic cleavage into the active di-chain. Purified samples were resolved by PAGE and visualised with SafeStain. Percentage purity and activation were determined by densitometry. Western blot analysis of rBoNT/B (B) and rBoNT/$B_{(S201P)}$ (C), using antibodies specific for LC/B (α LC/B) or H_C/B (α H_C/B), confirm the presence of the parent di-chain at ~150 kDa ("oxidised") and light and heavy chains at ~50 kDa and ~100 kDa, respectively ("reduced").

BoTest cell-free light chain activity assay (Figure 2)

- The light chain activities of rBoNT/B and rBoNT/$B_{(S201P)}$ were assessed using the BoTest (Biosentinel, Wisconsin, US).
- BoTest Reporter, VAMP-2(33-94), flanked by N-terminal cyan fluorescent protein and C-terminal yellow fluorescent protein was combined with reduced rBoNT/B or rBoNT/$B_{(S201P)}$ (1.25 nM – 0.5 pM) in a final volume of 100 µL/well.
- Plates were incubated at 30 °C for 18 hr. Fluorescence emission at 485/20 nm and 528/20 nm following excitation at 440/40 nm was measured using a BioTek Synergy HT plate reader.



Toxin	Potency (pIC$_{50}$)	% Relative Potency to rBoNT/B	*n*
rBoNT/B	10.14 ± 0.02	100	3
rBoNT/$B_{(S201P)}$	10.80 ± 0.03***	363.7 ± 51.0	3

Figure 2. The light chain activities of rBoNT/B and rBoNT/$B_{(S201P)}$ were assessed in the BoTest cell-free assay by their ability to cleave a fluorescently labelled VAMP substrate. The potency (pIC$_{50}$) of rBoNT/$B_{(S201P)}$ was significantly (P<0.001) greater than the pIC$_{50}$ of rBoNT/B (Paired t-test).

In vitro cell-based assays

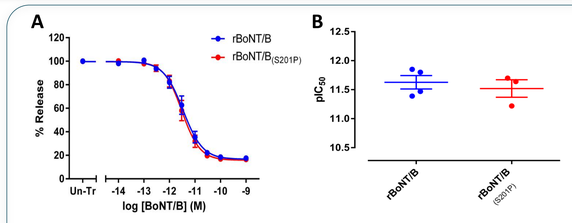
- Rat spinal cord neurons (SCN) were prepared from E15 Sprague-Dawley rat embryos; rat cerebral cortical neurons from E18 CD rat embryos (Charles River, UK). Neurons were cultured for up to 21 days.
- Neurons were treated with rBoNT/B or rBoNT/$B_{(S201P)}$ (SCN 1 nM – 10 fM; cortical neurons 10 nM – 1 pM) for 24 hrs. Toxins were removed and cells washed 3-times before use in assays.

[^{3}H]-Glycine release from SCNs (Figure 3)

- SCNs were loaded with 2 µCi/mL [^{3}H]-glycine (Perkin Elmer) in HEPES-buffered saline (HBS) for 60 mins at 35 °C. Basal and stimulated release of [^{3}H]-glycine were determined by incubating the cells for 5 min with HBS containing either low (3 mM) or high (60 mM) K^+ for basal and stimulated release, respectively. Superfusates were collected and radioactivity was quantified by liquid scintillation counting.

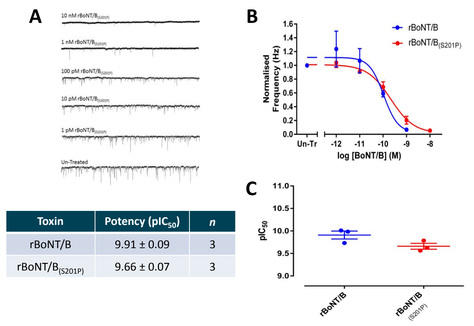
Cortical neuron patch clamp electrophysiology (Figure 4)

- Whole-cell recordings were performed in cortical neurons on glass coverslips at 35 °C using pipettes pulled from borosilicate glass capillaries with a resistance of 4-7 Mohm.
- Cultures were continuously perfused with artificial cerebrospinal fluid bubbled with 95% O_2/5% CO_2 (pH 7.4). mEPSCs were recorded at a holding potential of -70 mV and continuously perfused with 500 nM tetrodotoxin to block sodium channels, 10 µM bicuculline to block $GABA_A$ receptors and 1 µM CGP55845 to block $GABA_B$ receptors.



Toxin	Potency (pIC$_{50}$)	% Maximum Inhibition	*n*
rBoNT/B	11.63 ± 0.12	82.4 ± 0.7	4
rBoNT/$B_{(S201P)}$	11.52 ± 0.15	83.6 ± 1.1	3

Figure 3. A) The *in vitro* potencies of the BoNT/B toxins were compared in a cell-based neurotransmitter release assay using rat primary SCNs. Data were normalised to the [^{3}H]-glycine release obtained from un-treated cells (Un-Tr). B) The concentration of each toxin required for 50% maximal inhibition (pIC$_{50}$) of [^{3}H]-glycine release was determined from the fitted curves. rBoNT/B and rBoNT/$B_{(S201P)}$ were equipotent in inhibiting the release of pre-loaded [^{3}H]-glycine (p=0.589, un-paired t-test).



Toxin	Potency (pIC$_{50}$)	*n*
rBoNT/B	9.91 ± 0.09	3
rBoNT/$B_{(S201P)}$	9.66 ± 0.07	3

Figure 4. A) Cortical neurone cultures tested in patch-clamp electrophysiology assays to measure the inhibition of spontaneous neural network activity in cultures exposed to different concentrations of toxin. The frequency of mEPSCs was determined from 5-6 different cells for each toxin concentration. B) Data were normalised to the mEPSC frequency recorded from un-treated cells (Un-Tr). C) The concentration of each toxin required for 50% maximal inhibition (pIC$_{50}$) of mEPSC frequency was determined from the fitted curves. There was no significant difference between the potencies of rBoNT/B and rBoNT/$B_{(S201P)}$ (p=0.09, un-paired t-test).

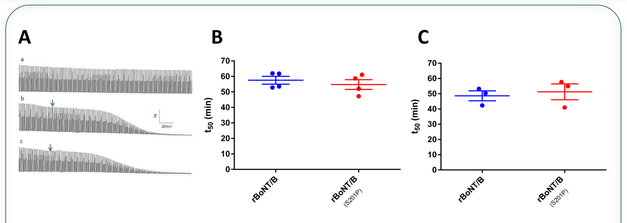
Ex vivo assays

C57Bl6 Mouse phrenic nerve hemi-diaphragm (Figure 5)

- The left phrenic nerve and hemi-diaphragm were removed and fixed to an electrode tissue holder suspended in an organ bath in Krebs-Henseleit buffer (KHB).
- The phrenic nerve was stimulated at 1 Hz, 100 ms duration. Contractile force of the diaphragm muscle was measured with isometric transducers.
- rBoNT/B or rBoNT/$B_{(S201P)}$ were added to the bath at 10 pM and the contractile response measured until full muscle paralysis had occurred.

C57Bl6 Mouse bladder strip (Figure 5)

- Two strips of detrusor muscle were dissected from one bladder and fixed onto an electrode tissue holder in an organ bath in KHB.
- Electrical field stimulation (EFS) at 10 Hz, with pulse duration of 0.3 ms and trains of 2 seconds duration every minute, was used to evoke contractions of the muscle.
- rBoNT/B or rBoNT/$B_{(S201P)}$ were added to the bath at 1 nM and the contractile response measured until 90% paralysis had occurred.



Toxin	Hemi-diaphragm Potency		*n*	Bladder Strip Potency		*n*
	(t_{10}, min)	(t_{50}, min)		(t_{10}, min)	(t_{50}, min)	
rBoNT/B	35.8 ± 0.9	57.5 ± 2.5	4	26.9 ± 2.7	48.6 ± 3.3	3
rBoNT/$B_{(S201P)}$	33.6 ± 2.1	54.8 ± 3.2	4	22.5 ± 1.6	51.2 ± 5.2	3

Figure 5. A) Following hemi-diaphragm tissue stabilisation, KHB (a), rBoNT/B (b) or rBoNT/$B_{(S201P)}$ (c) were added to the bath at the point denoted by the arrows. B) Data were normalised to the contractile strength prior to toxin addition. The time required to decrease the contractile strength by 10% (t_{10}) and 50% (t_{50}) was determined from the fitted curves. There was no significant difference in the potency of rBoNT/B or rBoNT/$B_{(S201P)}$ in the hemi-diaphragm (p=0.519, un-paired t-test). C) There were no significant differences in the t_{50} of rBoNT/B and rBoNT/$B_{(S201P)}$ inhibition of contraction of strips of detrusor muscle from the bladders of C57/Bl6 mice (p=0.689, un-paired t-test).

In vivo Digit Abduction Score (DAS) assay

- Mice were anaesthetized and injected in the gastrocnemius-soleus complex muscle of the right hind paw with a fixed volume of 20 µL rBoNT/B or rBoNT/$B_{(S201P)}$ (0.25 – 5.7 pg/mouse).
- DAS measurements were performed every day for 4 days and mice were weighed once a day up to 8 days.

Toxin	ED_{50} (pg)	DAS 4 (pg)	Lethal Dose (pg)	BW-10% (pg)	MTI (BW-10%/ED_{50})
rBoNT/B	1.3	3.5	3.5	2.3	1.8
rBoNT/$B_{(S201P)}$	1.3	5.2	5.2	3.0	2.3

- There were no significant differences between rBoNT/B and rBoNT/$B_{(S201P)}$ in either the effective dose required to achieve 50% the maximal effect (ED_{50}), or dose required to reach DAS 4.
- No significant differences (p>0.05) between the toxins were found in the safety parameters: either the dose causing 10% loss in body weight (BW-10%), or the Maximal Tolerated Index (MTI), a safety index calculated as the ratio of BW - 10%/ED_{50}.

Conclusions

- Full-length rBoNT/B incorporating the S201P mutation demonstrates enhanced activity in a cell-free light chain activity assay.
- The full length rBoNT/$B_{(S201P)}$ toxin is no more potent in biological assays *in vitro*, *ex vivo* and *in vivo*, than rBoNT/B.
- In order to enhance the efficacy of BoNT/B in humans, strategies other than enhancing light chain activity must be employed. For example, alteration in the binding characteristics of the neurotoxin.
- This study demonstrates that the limiting factor of toxin potency in complex biological systems is unlikely to be light chain activity.

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References

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