DUPLICATION OF NEURONAL BINDING DOMAINS DERIVED FROM CLOSTRIDIAL NEUROTOXINS GREATLY ENHANCE INTRANEURONAL DELIVERY OF THERAPEUTICS.

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Introduction

Neurologic diseases constitute 28% of the global disease burden and are expected to rise worldwide with the ageing of human populations. There is a need to develop new molecular systems that can deliver drugs specifically into neurons. Neuronal drug delivery must rely on agents that can recognize neurons with high specificity and affinity. Here we introduce a novel technology that utilizes duplicated botulinum binding domains, allowing neuronal targeting surpassing native botulinum neurotoxins.

Methods

We engineered binding domains of botulinum neurotoxins by attaching linker peptides and combined them to produce duplicated botulinum constructs. This resulted in high-affinity binding agents that can deliver imaging agents and large therapeutic enzymes into neuronal cytosol**.

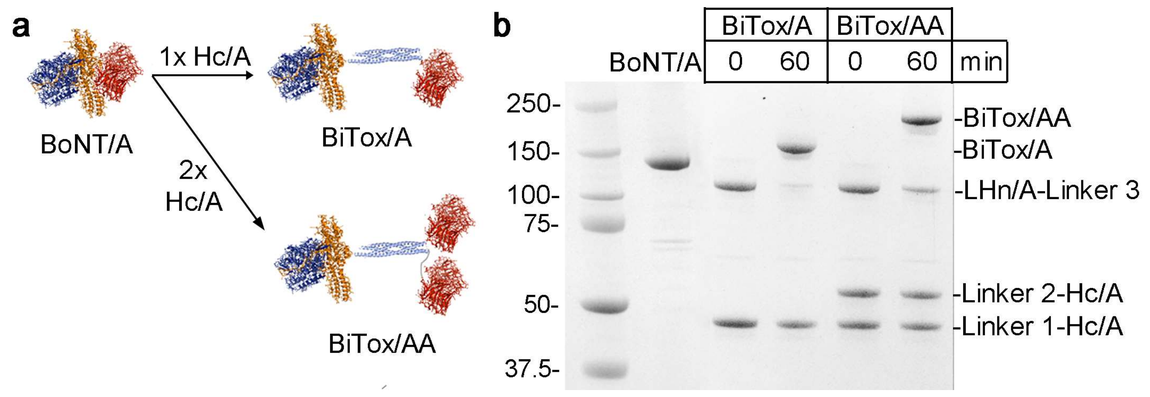

Figure 1 a. Schematic showing the structures of BoNT/A, BiTox/A and BiTox/AA with duplicated receptor-binding domain Hc/A. **b.** Coomassie-stained SDS-PAGE showing native BoNT/A and the formation of BiTox/A and BiTox/AA.

We also used recombinant fusion to create a single-molecule version of a duplicated botulinum neurotoxin type C (BoNT-2xHc/C), as a proof of concept for one-step production of duplicated botulinum neurotoxin molecules.

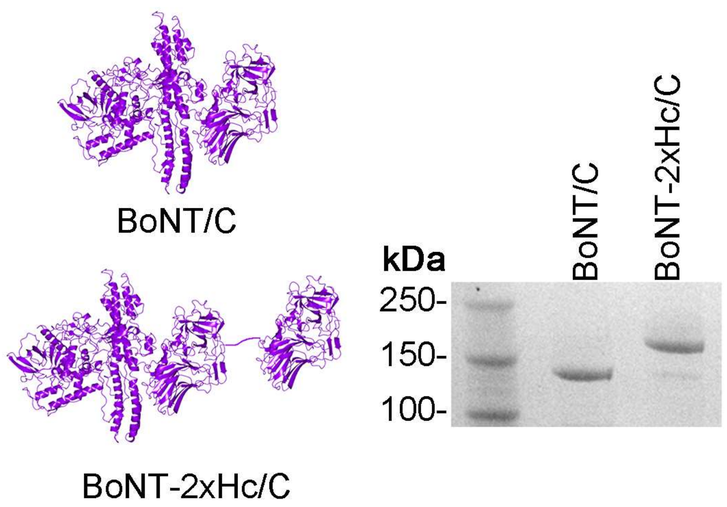

Figure 2 Structural diagram (left) and SDS-PAGE (right) showing the difference between BoNT/C and the novel BoNT-2xHc/C.

These agents were tested in differentiated human SiMa neuroblastoma cells for potency, using the proteolytic domain of botulinum neurotoxin type A and an immunoblotting assay to detect levels of its cleaved target, synaptosomal-associated protein (SNAP)-25.

As Botulinum type C has been found to induce apoptosis in differentiated neuroblastoma[1], Deep Blue cell viability assay was used to determine the level of cell death in cells treated with BoNT/C and our double-binding variant.

Finally, as BiTox/A has previously been found to reduce mechanical sensitivity in the Spared Nerve Injury (SNI) model of neuropathic pain in rats, while causing reduced paralysis compared to BoNT/A[2], rats were treated with either 2ng of BiTox/A or BiTox/AA 5 days after SNI surgery, and their mechanical sensitivity was measured by Von Frey Apparatus.

****Patent number:** WO2018109447A1 / GB201621111D0

Results

BiTox/AA cleaved SNAP-25 at lower concentrations than both the single-binding domain equivalent BiTox/A and native Botulinum toxin type A (BoNT/A), and has a faster onset of cleavage.

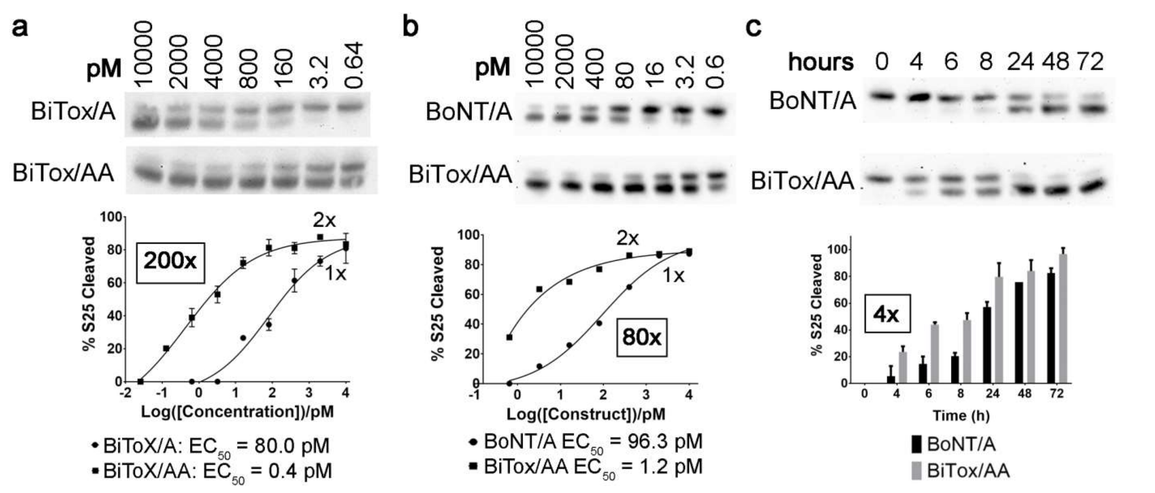

Figure 3 Immunoblots showing proportion of cellular SNAP-25 cleaved in differentiated neuroblastoma cells following application of BoNT/A variants. Lower graph represents quantification of the immunosignals, n=3 **a.** Titration comparing BiTox/A and BiTox/AA after 65h **b.** Titration comparing native BoNT/A with BiTox/AA after 65h. **c.** Time course of SNAP-25 cleavage after application of 2nM BoNT/A or 2nM BiTox/AA.

Cleavage of SNAP-25 by BoNT-2xHc/C was also more potent and had a faster onset time than the native Toxin equivalent, BoNT/C, and a higher degree of cell death was caused by the double-binding variant.

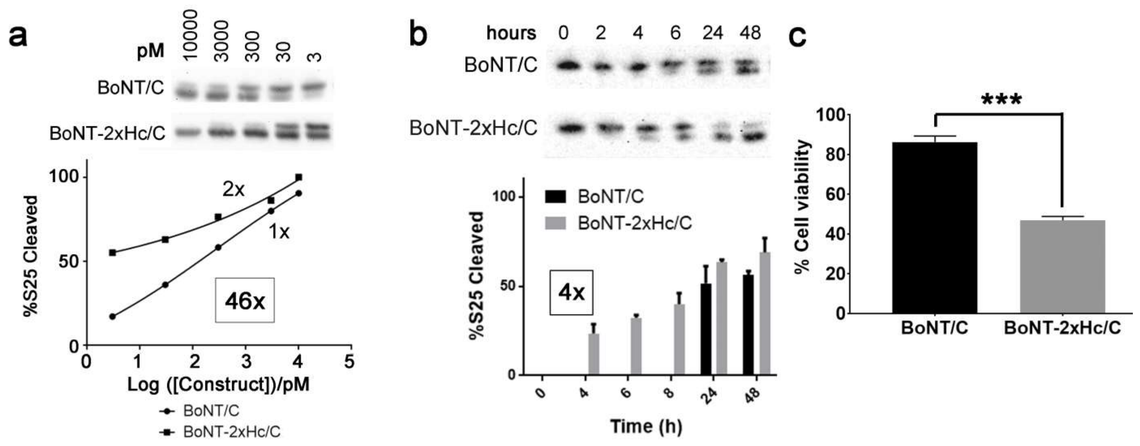

Figure 4 The additional binding domain of BoNT-2xHc/C allows for more potent and faster entry into differentiated SiMa neuroblastoma cells. **a +b.** Immunoblot showing proportion of cellular SNAP-25 following application of BoNT-2xHc/C and BoNT/C variants. Lower graph represents quantification of the immunosignals, n=3 **a.** Shows cleavage after 65h, **b** shows cleavage at 3nM concentration. **c.** Viability of differentiated SiMa neuroblastoma cells after 65h incubation with BoNT/C variants, normalised to cells treated with vehicle buffer, n=3. Significance determined using unpaired t-test. ***=<0.001.

BiTox/AA also caused reduced mechanical sensitivity in SNI model rats compared to BiTox/A, while still showing no signs of paralysis at the dose used.

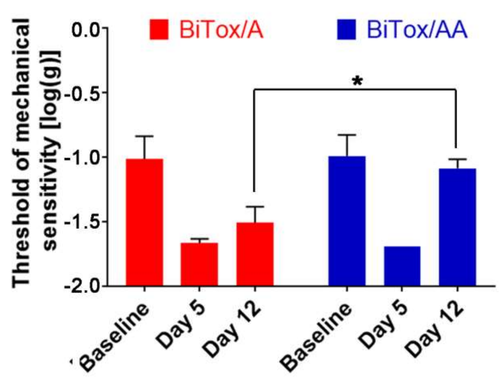

Figure 5 Mechanical sensitivity of SNI rats treated with BiTox/A and BiTox/AA. Graph shows baseline measurement (before surgery), Day 5 (after surgery, before injection of BiTox) and Day 12 (after surgery and injection of BiTox). Significance determined using one-way ANOVA, *=< 0.05

Ongoing Work

BiTox/AA is currently being trialed in other pain models, including migraine model, and we are testing the possibility of using these molecules to deliver other, non-clostridial, enzymes into neuronal cytosol.

Bibliography

[1]Rust et al. Oncotarget. 2016 May 31; 7(22): 33220–33228
[2] Mangione et al. Pain. 2016 May; 157(5): 1045–1055.