Design of modified botulinum toxin A variants with a shorter persistence of paralysis

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Introduction

- Botulinum neurotoxins (BoNTs) are the most toxic proteins known to humans. All seven known BoNT serotypes are synthesized as a single polypeptide chain of about 150 kDa.[1]
- Both BoNT/A and BoNT/B are used as pharmaceuticals. Given the long persistence of muscle paralysis induced by both BoNT/A and BoNT/B, these two serotypes are only suitable for indications that benefit from a duration of therapeutic effect between 3 and 6 months after injection.
- Today, there is no biological substance available to efficiently treat conditions where a significantly shorter duration of effect (3–6 weeks) is required.[2] BoNT/E and BoNT/F show a suitable therapeutic advantage in this respect, but are less potent than BoNT/A.[3]
- In this work, we focused on the modification of BoNT/A to synthesize a modified neurotoxin with a shortened persistence and a high specific potency of paralysis.
- Several studies strongly support the hypothesis that the remarkable duration of the paralytic effect of BoNT/A primarily results from retention of the active protease domain (light chain [LC]) within the nerve terminals.[4]
- Therefore, strategies to develop BoNT/A-based therapeutics with customized, shorter duration of therapeutic effect should modify the toxin in such a way that the intracellular half-life of its protease domain is shortened. The C-terminus contributes to the solubility, stability, and catalysis of the LC.[5]
- With our modifications, we therefore decided to focus on the C-terminal part of LC/A in order to achieve this challenging goal.

Methods

Preparation of BoNT/A variants

- For expression of BoNT/A in *E. coli*, codon-optimized gene constructs were cloned into pET29c. These variants contain C-terminal fused His6- and Strep-affinity tags that can be cleaved after protein purification via thrombin.
- For site-directed mutagenesis, a standard QuikChange® protocol was applied. Expression of recombinant BoNT (rBoNT)/A variants was performed in Riesenberg medium.
- Protein purification was performed using a three-step purification protocol:
  - immobilized-metal affinity chromatography
  - Strep-affinity chromatography
  - size-exclusion chromatography
- BoNT/A preparations were activated with thrombin yielding >99% of di-chain toxin. Toxin concentrations were determined by enzyme-linked immunosorbent assay measurements as described before.
- Modified BoNT/A constructs were expressed recombinantly in *E. coli* as single chain (SC) proteins. The specific mutations in the two modified toxin constructs (MCT3 and MCT4) are shown in **Table 1**. Treatment with thrombin cleaves the protein between LC and heavy chain (HC). Furthermore, thrombin cleaves the affinity tags at the C-termini of the protein.

Table 1. Overview of synthesized BoNT/A variants

Acronym	Mutations
MCT3	T420E F423M Y426F
MCT4	T420R F423M Y426F

BoNT/A, botulinum neurotoxin type A.

In silico analysis

- Mutations at the C-terminal region of BoNT/A LC were engineered with Schrödinger's biologics modeling platform BioLuminate (BioLuminate version 2.6, Schrödinger, LLC, New York, NY, United States).
- BioLuminate's automated residue scanning tool predicts relative stabilities as well as changes in solvent-accessible surface area, pKa, and hydropathy.

Digit abduction score (DAS) assay

- Groups of four mice were injected into the gastrocnemius muscle of the right hind limb with the indicated amounts of test substance (**Figure 1**).
- 0.6 U (3 pg) of incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals GmbH, Germany; BoNT/A wild type [WT]) was used as positive control.
- Local paralysis was assessed by a DAS for the injected limb.
- The toe spread was recorded according to a previously established protocol by Aoki,[6] ranging from 0 (normal toe spread) to 4 (maximum paralysis).
- The DAS was determined at 24-hourly intervals after toxin injection.

Voluntary mouse running assay (MRA)

- MRA was carried out using REVOLYZER® (preclinics GmbH, Germany) systems.
- The connected computer measured running distance evaluated as a half-hourly average. These data were recorded at daily intervals.
- One group was used as positive control and injected with incobotulinumtoxinA at 0.6 U (3 pg).
- To apply adequate dosages, the test substance was applied to achieve paralysis of 80–90% (i.e. 10–20% residual running activity), as aspired in the positive control group. The test substance was administered via intramuscular injections of 20 µL into the gastrocnemius muscle of the right hind limb.[3]

Results

- Results for the modified toxin constructs, MCT3 and MCT4, of purification (1) and after activation (2) are shown in **Figure 2**.

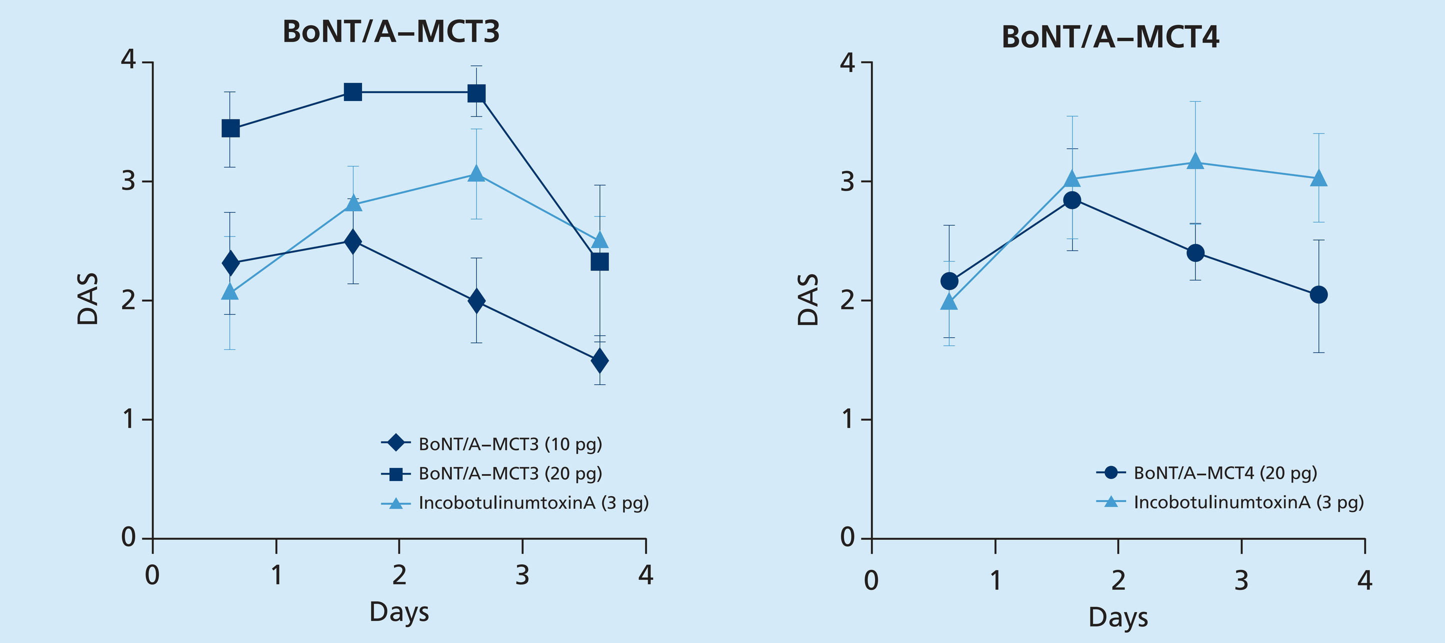
DAS assay

- Modified toxin constructs MCT3 and MCT4 of recombinant BoNT/A were analyzed in the DAS assay and the results are shown in **Figure 1**. The scoring results for MCT3 and MCT4 are indicative of a shorter duration of action. Notably, an approximate 7-fold higher dose is needed for equipotency to incobotulinumtoxinA (BoNT/A WT).

MRAs

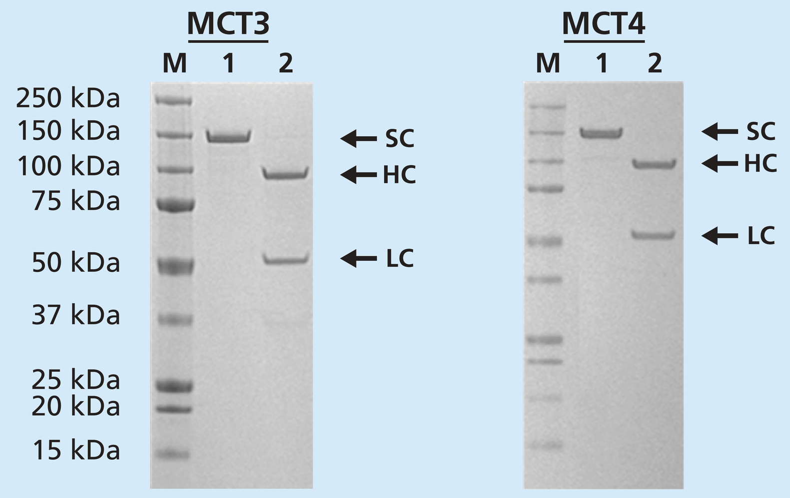
- Paralytic effects of recombinant BoNT/A variants MCT3 and MCT4 were analyzed in the MRA, and the results are shown in **Figure 3**. IncobotulinumtoxinA (BoNT/A WT) was used as an internal positive control. The negative control group consisted of mice that were not injected with any substance. Results for incobotulinumtoxinA represent the mean and standard deviation of 16 individual assays. MCT3 showed a largely shortened duration of the paralytic effect, with a sharp minimum of running activity on Day 2. The duration of paralytic effect of MCT4 was somewhat shortened compared to that of incobotulinumtoxinA.

Figure 1. Results of DAS assays


BoNT/A–MCT3
BoNT/A–MCT4
DAS
Days
BoNT/A–MCT3 (10 pg)
BoNT/A–MCT3 (20 pg)
IncobotulinumtoxinA (3 pg)
BoNT/A–MCT4 (20 pg)
IncobotulinumtoxinA (3 pg)

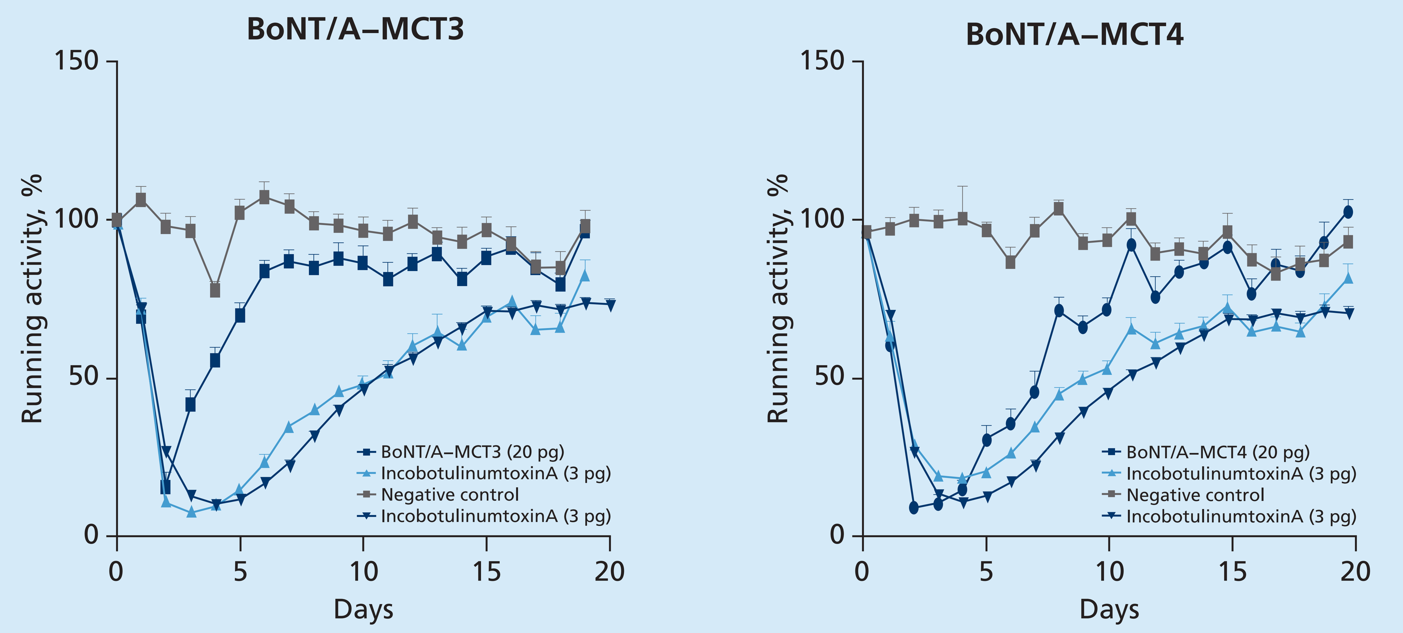
BoNT/A, botulinum neurotoxin type A; DAS, digit abduction score.

Figure 2. Activation of recombinant BoNT/A variants used in this study


MCT3
MCT4
M 1 2
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
SC
HC
LC

BoNT/A, botulinum neurotoxin type A; HC, heavy chain; LC, light chain; M, molecular weight marker; SC, single chain.

Figure 3. Results of voluntary MRAs


BoNT/A–MCT3
BoNT/A–MCT4
Running activity, %
Days
BoNT/A–MCT3 (20 pg)
IncobotulinumtoxinA (3 pg)
Negative control
IncobotulinumtoxinA (3 pg)
BoNT/A–MCT4 (20 pg)
IncobotulinumtoxinA (3 pg)
Negative control
IncobotulinumtoxinA (3 pg)

The paralysis effects of recombinant BoNT/A variants (dark blue squares [MCT3] and circles [MCT4]) were analyzed in this assay. IncobotulinumtoxinA (BoNT/A WT; light blue triangles) was used as an internal positive control. The negative control group comprised mice that were not injected with any substance (grey squares). IncobotulinumtoxinA (average of 16 assays; dark blue triangles) consists of mean and standard deviation of 16 individual assays.
BoNT, botulinum neurotoxin; MRAs, mouse running assays; WT, wild type.

Conclusions

- Serotypes of botulinum neurotoxins are hallmarked by a different onset and duration of effect. BoNT/A and BoNT/E, in particular, differ markedly in their kinetic profile. BoNT/E shows a fast onset but a short duration of paralysis, whereas the onset of BoNT/A is slower and the effect lasts markedly longer.
- In this work we were able to design, for the first time, BoNT/A variants with modifications at the C-terminus of the LC that show a substantially modified kinetic profile in comparison to WT BoNT/A and a high similarity to that of BoNT/E *in vivo*.
- The C-terminus seems to play a key role, by controlling the course of paralysis and the onset of effect.
- We have shown that a genetically modified BoNT/A can close the gap between the long-lasting WT form of BoNT/A available as complex form (onabotulinumtoxinA and abobotulinumtoxinA)[7] and pure neurotoxin (incobotulinumtoxinA)[7] and the short-acting BoNT/E with respect to duration of action.
- An additional advantage of the short-lived MCT3 and MCT4 mutants is that they show only a slightly lower potency compared with WT BoNT/A. The low protein dose needed offers the opportunity of a repeated use of the new designed proteins.

References

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Acknowledgments

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