Properties of a chimeric BoNT/A1–A4

Daniel Scheps, Michael Schmidt, Marcel Jurk, Manuela López del Paz, **Jürgen Frevert**

Merz Pharmaceuticals GmbH, Potsdam, Germany

Botulinum Toxin Research
Merz Pharmaceuticals

Summary

- The specific potency of the activated botulimum neurotoxin (BoNT)/A1–A4 chimera (light chain $[LC]_{A4}$–heavy chain $[HC]_{A1}$) was reduced when compared with BoNT/A1, but markedly higher than the specific potency of wild type (WT) BoNT/A4 according to the values reported in the literature.
- In comparison with WT BoNT/A1 (incobotulinumtoxinA [Xeomin®]; Merz Pharmaceuticals GmbH; Germany), the chimera showed a later onset of effect in the digit abduction score (DAS) assay and in the mouse running assay (MRA). The recovery from the paralytic effect was also shifted to a slightly later time point when assessed in the MRA.
- Conclusions:
  - In contrast to BoNT/A4, the chimeric BoNT/A1–A4 could be purified from *Escherichia coli*.
  - The pharmacological properties of the chimeric protein differed from the parent neurotoxins BoNT/A1 and BoNT/A4.
  - It can be hypothesized that the low specific potency of BoNT/A4 is based on the low binding affinity of its heavy chain.

Background

- Currently, 10 different subtypes of BoNT type A are known.[1] Pharmacological analysis of some of the subtypes has revealed that they exhibit different enzymatic activities and receptor binding *in vitro* and different potencies in animal assays.[2–4]
- BoNT/A1 and BoNT/A2 are ~95% homologous, and possess similar enzymatic acitivities. BoNT/A3 and BoNT/A4 are 81% and 88% homologous to BoNT/A1, respectively, show a similiar affinity to the substrate synaptosome associated protein 25 (SNAP25) but have a different capacity for SNAP25 cleavage and do not show autocatalytic cleavage.[2]
- A truncated LC of BoNT/A4 was expressed and purified from *E. coli* and showed an enzymatic activity approximately 100-fold lower than the LC of BoNT/A1. Mutation of a single amino acid (I264R) almost restored enzymatic activity.[2]
- A second mutation (L260F) was introduced to improve the solubility but had no effect on the enzymatic activity.[2]
- Because expression of the holotoxin in *E. coli* could not be achieved, BoNT/A4 was expressed in a nontoxigenic *Clostridium botulinum* expression system.[3]
- The specific potency of recombinant BoNT/A4 (rBoNT/A4) was ~1.0×10^5 LD_{50}/U/mg; i.e. ~1000-fold lower than the specific potency of BoNT/A1. Surprisingly, the specific potency of the BoNT/A4–L260F I264R mutant was not increased (1.1×10^5 LD_{50}/U/mg).[3]
- In contrast, the specific *in vitro* activity (BoTest®) of rBoNT/A4 was only ~6-fold less than that of BoNT/A1, and that of the BoNT/A4–L260F I264R mutant was only ~2-fold less compared with BoNT/A1.[3]
- The *in vitro* activity of WT BoNT/A4, and of the mutant, in cultured human neurons mirrored the *in vivo* potency and was ~1000-fold lower than the potency of BoNT/A1.[4]
- Analysis of rBoNT/A4 in primary spinal cord cells revealed a long persistence of the LC, showing that SNAP25 was still cleaved by ~75% after 9 months.[3]
- *In vivo* BoNT/A4 showed a slightly later onset of effect (DAS assay), and a recovery similar to BoNT/A1 (Rotarod assay).[5]

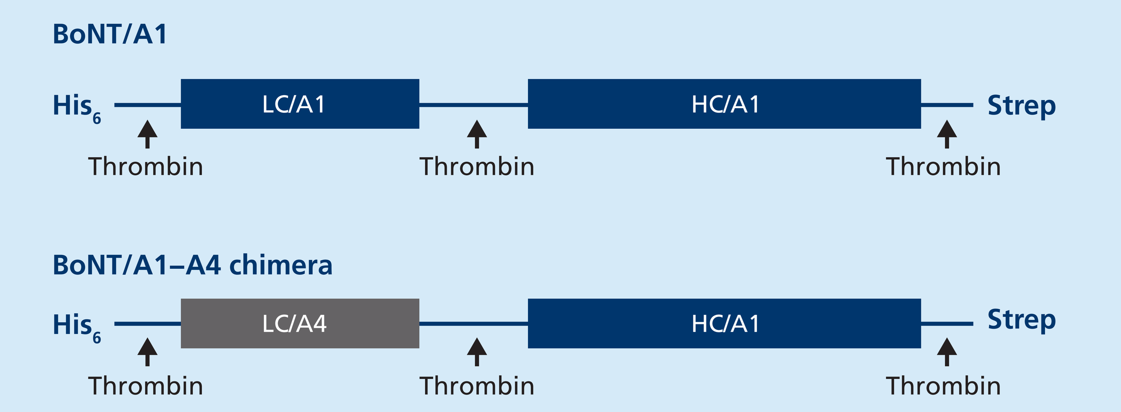
Methods

- In order to construct a plasmid for the expression of BoNT/A1–A4 in *E. coli*, a synthetic gene (codon optimized, Thermo Scientific) with the corresponding LC/A4 and HC/A1 gene sequence was cloned into pET29c.
- The construct comprised N-terminal-fused His_6- and C-terminal strep-affinity tags, which were cleaved off via thrombin after protein purification. Expression of rBoNT/A variants was performed in Riesenberg media.
- The recombinant BoNT was purified by immobilized metal affinity chromatography using a HisTrap HP 1-mL column (GE Healthcare), followed by Strep-Tactin® affinity chromatography as described (IBA GmbH) and finally by size exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare).
- The protein was >98% pure as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). BoNT/A preparations were activated with thrombin (Merck Millipore; 8 U/1 mg BoNT) for 24 hours at 20 °C yielding >99% of di-chain toxin. After cleavage, the protease was removed by applying Strep-Tactin® affinity chromatography as described above.
- The relative potency was determined in the DAS assay[6] with four mice/dose using 0.6 U of incobotulinumtoxinA as standard.
- Onset of paralysis and duration of effect was determined in the MRA as described previously,[7] with 10 animals/dose. An incobotulinumtoxinA dose of 0.6 U was used as standard, achieving a reduction in running distance of ~90%.

Results

- In contrast to WT BoNT/A4, the chimera (composition described in **Figure 1**) could be expressed in *E. coli*.

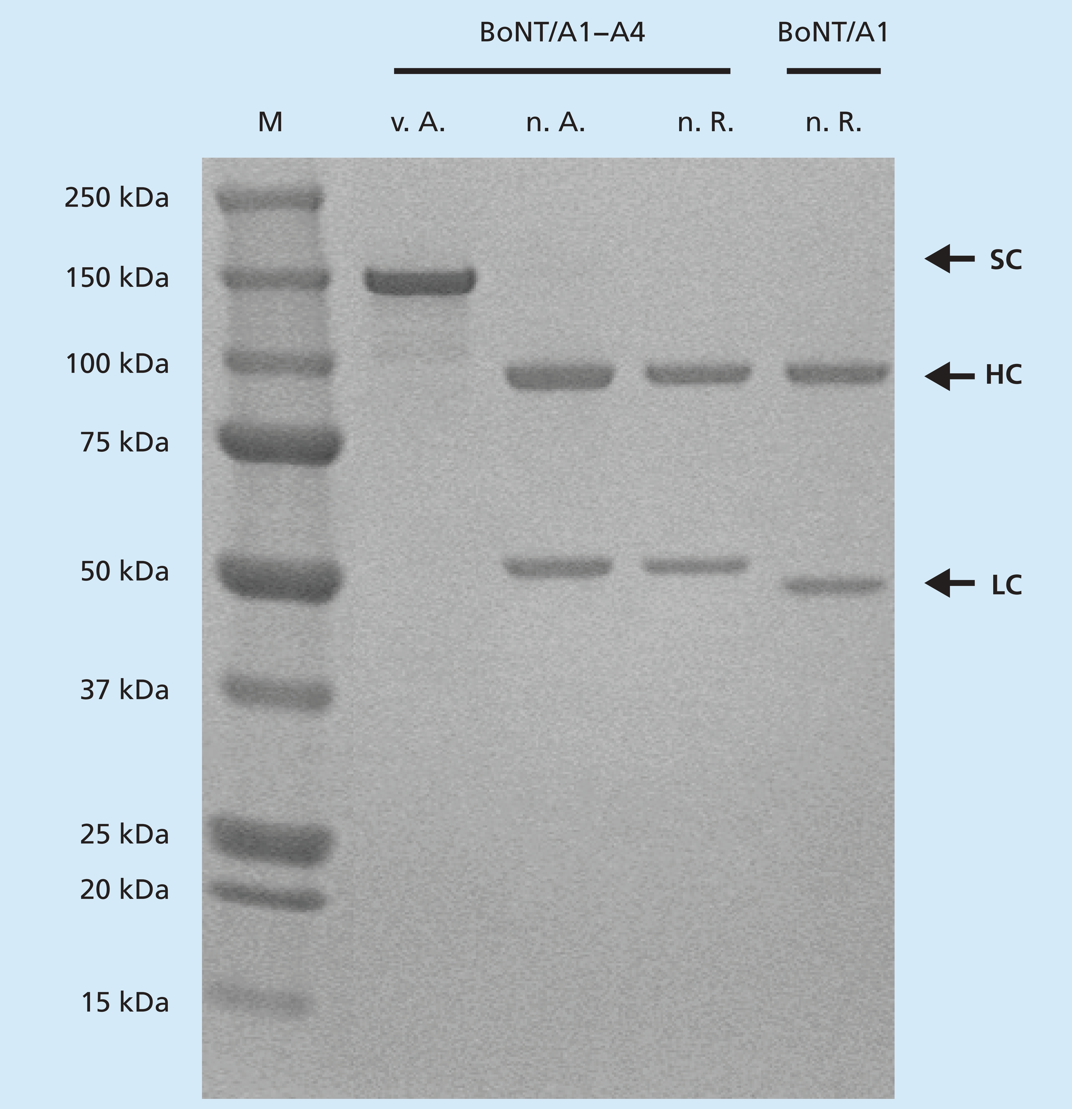
Figure 1. Composition of the BoNT/A1–A4 chimera


BoNT/A1
His6
LC/A1
HC/A1
Strep
Thrombin
Thrombin
Thrombin
BoNT/A1–A4 chimera
His6
LC/A4
HC/A1
Strep
Thrombin
Thrombin
Thrombin

The LC of BoNT/A4 was fused with the HC of BoNT/A1 linked with a thrombin cleavage site for post-translational activation. To facilitate purification, an N-terminal His tag and a C-terminal strep tag were fused, which were cleaved off during activation.
BoNT, botulinum neurotoxin; HC, heavy chain; LC, light chain.

- After thrombin cleavage, the purified construct was free of tags that could potentially influence *in vivo* assays (**Figure 2**).

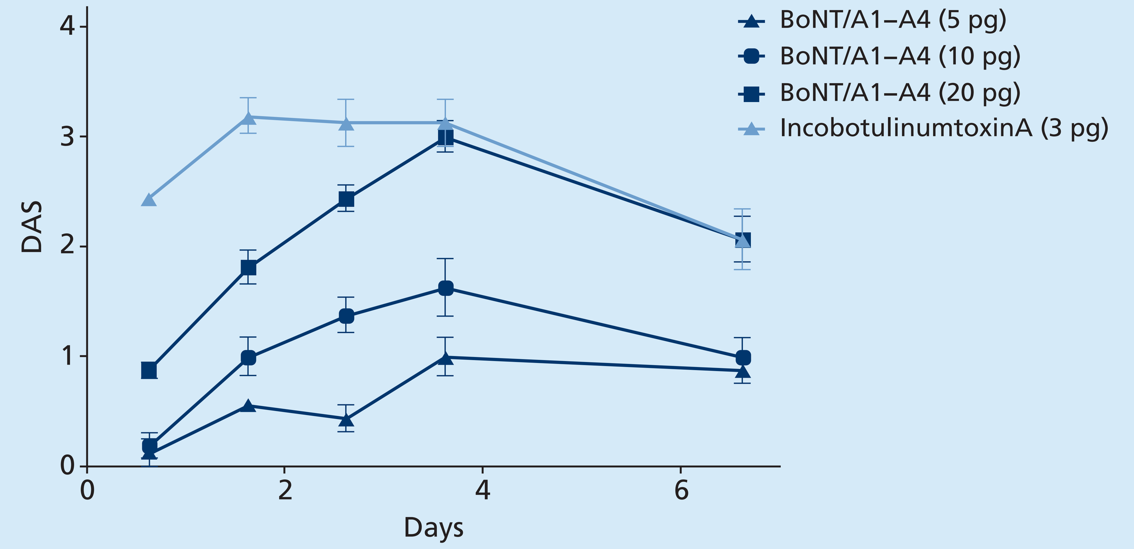
Figure 2. SDS-PAGE of purified BoNT/A1–A4 after activation with thrombin


BoNT/A1–A4
BoNT/A1
M
v. A.
n. A.
n. R.
n. R.
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
SC
HC
LC

BoNT, botulinum neurotoxin; HC, heavy chain; LC, light chain; M, molecular weight marker; n.A., after cleavage with thrombin; n.R., after purification of activated BoNT/A1–A4 (removal of thrombin); SC, single chain; v.A., SC BoNT/A1–A4.

- The specific potency assessed in the DAS assay (**Figure 3**) seemed to be reduced by 6–7-fold compared with WT BoNT/A1 (incobotulinumtoxinA).
  - In comparison with WT BoNT/A1, the peak effect of paralysis was reached later.

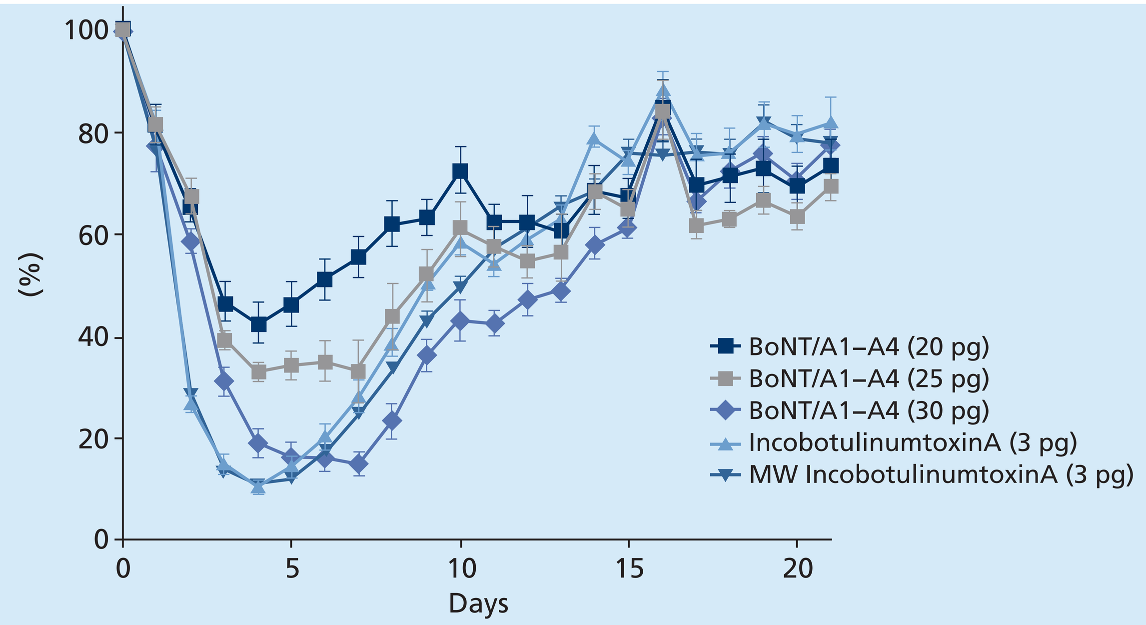
Figure 3. Analysis of BoNT/A1–A4 in the DAS assay


DAS
Days
0
1
2
3
4
0
2
4
6
BoNT/A1–A4 (5 pg)
BoNT/A1–A4 (10 pg)
BoNT/A1–A4 (20 pg)
IncobotulinumtoxinA (3 pg)

5, 10 or 20 pg of BoNT/A1–A4 in 20 µL were injected into the gastrocnemius of mice (right hind limb). incobotulinumtoxinA (3 pg) was used as a standard. The digit abduction was scored according to a 5-score scale.[6]
BoNT, botulinum neurotoxin; DAS, digit abduction score.

- The later onset of paralysis was confirmed in the MRA (**Figure 4**).
- The equipotent dose of the chimera in the MRA was ~10-fold higher compared WT BoNT/A1:
  - 10 times more protein had to be applied to achieve the same degree of paralysis (90%) as WT BoNT/A1.
  - The equipotent dose of the chimera (30 pg) caused maximum paralysis after 7 days, markedly later than with WT BoNT/A1 (Day 4).

Figure 4. Analysis of BoNT/A1–A4 in the MRA[7]


(%)
Days
0
20
40
60
80
100
0
5
10
15
20
BoNT/A1–A4 (20 pg)
BoNT/A1–A4 (25 pg)
BoNT/A1–A4 (30 pg)
IncobotulinumtoxinA (3 pg)
MW IncobotulinumtoxinA (3 pg)

20, 25 or 30 pg of BoNT/A1–A4 in 20 µL were injected into the gastrocnemius of mice (right hind limb). The running distance was monitored in daily intervals. IncobotulinumtoxinA (3 pg) was used as a standard for comparison.
BoNT, botulinum neurotoxin; MRA, mouse running assay; MW, mean of 17 assay runs of IncobotulinumtoxinA (3 pg).

- The recovery profile was similar to WT BoNT/A1 but with a parallel shift to a later recovery. The total time-span of paralysis did not seem to be different from that of WT BoNT/A1.

Conclusions

- Whereas WT BoNT/A4 showed a specific potency ~1000-fold lower than BoNT/A1, the chimera BoNT/A1–A4 was characterized by a markedly higher specific potency in the DAS assay and the MRA (only ~10-fold lower than BoNT/A1).
- Since it was shown that a marked enhancement of the protease activity in the BoNT/A4–L260F I264R LC mutant did not increase the specific potency of the holotoxin *in vivo*, one can conclude that the potency is markedly determined by the HC, presumably by the binding affinity to synaptic vesicle protein SV2 and/or gangliosides.
- It was demonstrated that WT BoNT/A4 had a later onset of effect,[5] which was also the case for the chimera.
- One could hypothesize that the later onset is controlled by the properties of the LCs (low enzymatic activity). The different HCs of BoNT/A1 versus BoNT/A4 do not seem to influence this effect – although the impact of interplay between HC and LC during translocation cannot be excluded.
- The BoNT/A1–A4 chimera did not show a prolongation of paralysis when compared with BoNT/A1[5] (only a shift) although *in vitro* studies demonstrated a long persistence of BoNT/A4 in neuronal cell cultures.[4]

References

1. Rossetto O et al. Nat Rev Microbiol 2014, 12: 535–549.
2. Henkel JS et al. Biochemistry 2009; 48: 2522–2528.
3. Bradshaw M et al. Appl Environ Microbiol 2014; 80: 7415–7422.
4. Whitemarsh RC et al. PLoS One 2014; 9: e90252.
5. Pellett S et al. Toxicon 2015; 107: 37–42.
6. Keller JE. Neuroscience 2006; 139: 629–637.
7. Aoki KR. Toxicon 2001; 39: 1815–1820.

Acknowledgements

This study was sponsored by Merz Pharmaceuticals GmbH, Frankfurt am Main, Germany. Editorial support was provided by Complete Medical Communications, funded by Merz.

Presented at TOXINS 2017, Madrid, Spain, 18–21 January 2017