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Design of modified botulinum neurotoxin A1 variants with a shorter persistence of paralysis and duration of action

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ABSTRACT

Botulinum neurotoxins (BoNTs) are classified by their antigenic properties into seven serotypes (A-G) and in addition by their corresponding subtypes. They are further characterized by divergent onset and duration of effect. Injections of low doses of botulinum neurotoxins cause localized muscle paralysis that is beneficial for the treatment of several medical disorders and aesthetic indications. Optimizing the therapeutic properties could offer new treatment opportunities.

This report describes a rational design approach to modify the pharmacological properties by mutations in the C-terminus of BoNT/A1 light chain (LC). Toxins with C-terminal modified LC's displayed an altered onset and duration of the paralytic effect *in vivo*. The level of effect was dependent on the kind of the mutation in the sequence of the C-terminus. A mutant with three mutations (T420E F423M Y426F) revealed a faster onset and a shorter duration than BoNT/A1 wild type (WT).

It could be shown that the C-terminus of BoNT/A1-Lc controls both onset and duration of effect. Thus, it is possible to create a mutated BoNT/A1 with different pharmacological properties which might be useful in the therapy of new indications. This strategy opens the way to design BoNT variants with novel and useful properties.

1. Introduction

Botulinum neurotoxins (BoNTs) are the most toxic proteins for humans known so far. BoNTs are synthesized *via* anaerobic fermentation by *Clostridium botulinum* but in rare cases also by *Clostridium baratii* or *Clostridium butyricum* (Montal, 2010). They are classified into seven immunological distinct serotypes (A-G), A, B and E being responsible for most human intoxications (Rossetto et al., 2014). Each serotype is further categorized into different subtypes on the basis of their amino acid sequences which are well described by Rosetto et al. (Rossetto et al., 2014).

All BoNTs are synthesized as a single polypeptide chain of about 150 kDa. This chain is processed by posttranslational proteolytic cleavage at a loop to yield the active toxin, which consists of distinct N- and C-terminal light and heavy chain, respectively (Pirazzini et al., 2017). Light and Heavy chain (LC and HC) are kept together by a long peptide belt, non-covalent interactions and by a disulfide bond.

The LC is a zinc metalloprotease with a typical metal coordination motif (HExxH) which cleaves specifically one of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins that are necessary for neurotransmitter exocytosis (Rossetto et al., 2014). Cleavage causes the flaccid paralysis observed following exposure to BoNT. Two different functional subunits form the HC: the H_N subunit (at the N-terminus of the HC), responsible for translocation of the LC into the cytosol of the neuron; and the H_C subunit (at the C-terminus of the HC), required for neuronal cell receptor recognition and presynaptic binding.

The Food and Drug Administration (FDA) has approved several therapeutics for the treatment of various neurologic disorders like cervical dystonia (spasmodic torticollis), blepharospasm, different forms of spasticity, migraine as well as urologic disorders based on two toxin serotypes: A, particularly the A1 subtype (BoNT/A1 - abo-, ona-, and incobotulinumtoxinA) and B, specifically the B1 subtype (BoNT/B1 - rimabotulinumtoxinB). In addition, the application of BoNT/A-containing therapeutics in aesthetic medicine to treat facial wrinkles (e.g. glabella lines and crow's feet) is a strongly growing market. In this field BoNT/A is already the most popular product of all cosmetic procedures worldwide (http://www.isaps.org/news/isaps-global-statistics).

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Given the long persistence of the paralysis induced by both BoNT/A1 and slightly shorter by BoNT/B1, these two subtypes are suitable only for indications that benefit from a duration of therapeutic effect between 3 and 6 months after injection (Frevert, 2015). No other protein drug shows such a long duration of activity. Although a shorter duration of action has been shown in humans for BoNT/E1 (Eleopra et al., 1998), until now there is no neurotoxin commercially available to efficiently treat conditions where a significantly shorter duration of effect (3–6 weeks) is required. Especially in the fields of orthopedics and rehabilitation a toxin with a shortened persistence would be highly appreciated for additional treatment options (Pirazzini et al., 2017; Rossetto et al., 2014). Although BoNT/E, BoNT/A3 and BoNT/F show a suitable therapeutic window in that respect, they are significantly less potent compared to BoNT/A1 (Eleopra et al., 1998; Kauffman et al., 1985; Keller et al., 2004; Pellett et al., 2015; Sellin et al., 1983). Therefore, an engineered BoNT/A1 with a shortened duration of effect could be an attractive alternative to the aforementioned toxins. Due to that fact we focused in this work on the modification of BoNT/A1 to synthesize a modified neurotoxin with a shortened duration of paralysis.

Understanding the mechanisms underlying the extraordinary persistence of BoNT/A1 in neurons would allow the rational modification of the toxin in order to fine-tune the duration of action depending on the therapeutic indication. Literature data strongly support the hypothesis that the remarkable duration of the paralytic effect of BoNT/A1 primarily results from retention of the LC within the nerve termini (Shoemaker and Oyler, 2013). Therefore, strategies to develop BoNT/A1-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 reduced to a certain extent. Just recently it was demonstrated that the long duration of BoNT/A1 is determined by the effect of the deubiquitinating enzyme VCIP135 (Tsai et al., 2017). By inhibition of this enzyme the duration of effect could also be shortened.

With this aim, we focused our design efforts on the LC of BoNT/A1 (LC/A1). Based on the contribution of the C-terminus of LC/A1 to its solubility, stability and catalysis (Baldwin et al., 2004), we hypothesized that modifications of this part of the molecule should influence its persistence within the neuron. Indeed, by mutating amino acids at the C-terminus of the light chain, we were able to create a BoNT/A1 variant with a modified LC which exhibits a significantly shorter duration of action than the wild-type toxin. This result highlights the prominent role of the C-terminus of the LC of BoNT/A1 in controlling the duration of effect of the toxin. In addition, the high potency of the wild-type BoNT/A1 could be retained in the modified toxins, hence rendering these variants useful in the therapy of new indications.

2. Materials and methods

2.1. Protein modeling and design

Protein coordinates were retrieved from the RCSB Protein Data Bank (PDB; http://www.rcsb.org/pdb/) (Berman et al., 2000). Among the structures of the Zn^{2+}-bound catalytic domain of BoNT/A1 deposited in the PDB, 3BON with a resolution of 1.2 Å (residues Met1 – Phe425) (Silvaggi et al., 2008) was chosen for modeling and design purposes. The SNAP25-bound state of the LC was modeled using the structure 1XTG (resolution 2.1 Å) (Breidenbach and Brunger, 2004). Modeling of the full-length toxin was performed using the structure 3BTA (resolution 3.2 Å) (Lacy et al., 1998).

Modeling and mutant design was performed with Schrödinger's molecular modeling suite [Schrödinger LLC, New York, NY, USA, 2014–2015]. A comprehensive description of the methodology can be found in the supplementary material (SM). In brief, PDB structures were prepared prior to any other modeling steps using Schrödinger's protein preparation wizard. The C-terminal fragment of the LC/A1 that cannot be observed in any of the currently available X-Ray structures was modeled *de novo* up to Lys438 (mature full-length LC/A1) (Krieglstein et al., 1994). Prior to mutant design, the modeled structures were refined and analyzed by Molecular Dynamics (Bowers et al., 2006). Mutations at the C-terminal LC/A1 positions Thr420, Phe423, Tyr426, and Leu429 were engineered with Schrödinger's biologics modeling platform BioLuminate (BioLuminate versions 1.6–1.8 for the single-point mutants, and version 2.6 for the four- and three-point mutants; Schrödinger, LLC, New York, NY, USA). The estimated change in relative stability of the protein upon mutation was used as read-out and criterion for the selection of mutants to be tested experimentally.

2.2. Molecular biology

For expression of BoNT/A1 in *E. coli* codon optimized gene constructs were cloned into pET29c (DNA and protein sequence in SM table TS1). These variants contain C-terminal fused His6-and Strep-affinity tags which can be cleaved off after protein purification *via* thrombin. In order to construct a plasmid for the expression of MCT5 a synthetic gene (Thermo Scientific) with the corresponding mutations of MCT5 (Thr420Arg Phe423Met Tyr426Arg Leu429Met) and restriction sites *Sbf*I and *Sca*I was used. For site directed mutagenesis of MCT1 (Thr420Arg), MCT2 (Lys427Tyr), MCT3 (Thr420Glu Phe423Met Tyr426Phe), MCT4 (Thr420Arg Phe423Met Tyr426Phe), MCT6 (Thr420Arg Phe423Y Y426Arg Leu429Met) and MCT7 (Thr420Arg Phe423Met Y426Arg Leu429Ile) standard QuikChange II XL (Agilent Technologies) protocol was applied. The corresponding primers and templates are listed in SM table TS2.

2.3. Protein expression and purification

Expression of rBoNT/A1 variants was performed in Riesenberg minimal medium with 50 μg/mL Kanamycin (Riesenberg et al., 1991). Cells were grown in shake flasks (37 °C, 175 rpm) until an OD_{600} of 1.5–2 was reached. For induction of protein expression 1 mM IPTG (Fermentas) was added to the *E. coli* culture. Protein synthesis was performed for 24 h (15 °C, 175 rpm). Cells were collected by centrifugation (5000 rpm., 20 min, 4 °C) and resuspended in His binding buffer pH 8.0 (50 mM Tris, 150 mM NaCl, 5 mM Imidazole) containing EDTA-free protease inhibitor complete (Roche Diagnostics). For the determination of endopeptidase activity and *in vivo* characterization, the different toxin variants were extracted and purified. Resuspended pellets were disrupted in 2–3 cycles by a French Press Cell Disrupter (Thermo Electron Corporation) at 4 °C. The resulting crude extracts were centrifuged (20,000 r.p.m., 30 min, 4 °C), and the supernatants with the soluble proteins were recovered. Protein purification was carried out by fast protein liquid chromatography (GE Healthcare) using a three step purification protocol. The first capture step was performed by IMAC using a HisTrap HP 1 mL column (GE Healthcare). Proteins were eluted (1 ml min-1 working flow) using a two-step protocol with His elution buffer (50 mM Tris, 150 mM NaCl, 400 mM Imidazole pH 8.0). The elution of the toxin proteins occurred at 400 mM Imidazole. In a further step a Strep-Tactin affinity chromatography was used as previously described (IBA GmbH). As an alternative instead of the second affinity chromatography, a cation exchange chromatography with a HiTrap SP HP 1 mL column (GE Healthcare, Freiburg, Germany) was used. The corresponding samples were diluted with SP binding buffer (50 mM Tris, pH 8.0) and eluted with