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In vitro potency determination of botulinum neurotoxin serotype A based on its receptor-binding and proteolytic characteristics

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ABSTRACT

Botulinum neurotoxins (BoNTs) inhibit the release of the neurotransmitter acetylcholine from motor neurons, resulting in highly effective muscle relaxation. In clinical and aesthetic medicine, serotype BoNT/A, which is most potent for humans, is widely used to treat a continuously increasing spectrum of disorders associated with muscle overactivity. Because of the high toxicity associated with BoNTs, it is mandatory to precisely determine the potency of every batch produced for pharmaceutical purposes. Here we report a new quantitative functional *in vitro* assay for BoNT/A. In this binding and cleavage (BINACLE) assay, the toxin is first bound to specific receptor molecules. Then a chemical reduction is performed, thereby releasing the light chain of BoNT/A and activating its proteolytic domain. The activated light chain is finally exposed to its substrate protein SNAP-25, and the fragment resulting from the proteolytic cleavage of this protein is quantified in an antibody-mediated reaction. The BoNT/A BINACLE assay offers high specificity and sensitivity with a detection limit below 0.5 mouse lethal dose (LD_{50})/ml. In conclusion, this new *in vitro* assay for determining BoNT/A toxicity represents an alternative to the LD_{50} test in mice, which is the "gold standard" method for the potency testing of BoNT/A products.

1. Introduction

Botulinum neurotoxins (BoNTs) are protein toxins that are produced by the ubiquitous bacterium *Clostridium botulinum*. An intoxication with BoNTs leads to botulism, a potentially fatal disease that is characterized by a severe flaccid paralysis. Estimated median lethal doses of 0.5–2 ng per kg body weight have been described for humans (World Health Organization, 2017; Arnon et al., 2001) and many animal species (Gill, 1982), which make BoNTs the most potent toxins known. Three of the seven BoNT serotypes, namely BoNT/A, BoNT/B, and BoNT/E, have frequently been associated with food-borne intoxications in humans (Arnon et al., 2001). In recent years, BoNTs have also gained considerable attention in a different context: Since the late 1980s, their muscle-relaxing effects have been exploited in clinical and aesthetic medicine (Chen, 2012). Some of the most important clinical indications for BoNTs include cervical dystonia, upper limb spasticities, urinary incontinence, strabismus, and hyperhidrosis. BoNT products are also used to treat wrinkles caused by contractions of the facial muscles. In addition to the muscle-paralyzing effects, which have been known for many years, antinociceptive properties of BoNTs have recently been described (Li and Coffield, 2016). Therefore, BoNTs are also under investigation for the treatment of diverse pain-related disorders (Mittal et al., 2016). Due to the continuously increasing spectrum of approved as well as off-label indications, the overall market for BoNT products is expanding rapidly. Most of the products used to date contain BoNT/A, which is the most effective BoNT serotype in humans. An approved drug based on serotype BoNT/B is also available, but shows a 30- to 100-fold lower therapeutic activity than the BoNT/A products (Bentivoglio et al., 2015).

In order to avoid toxic side effects, the potency of each BoNT batch produced for pharmaceutical or cosmetic applications has to be precisely determined before its release onto the market. For this purpose

Abbreviations: BINACLE, Binding and cleavage assay; BoNT, Botulinum neurotoxin; BSA, Bovine serum albumin; GT1b, Trisialoganglioside GT1b; H-chain, Heavy chain; HCcA, C-Terminal region of the BoNT/A H-chain; L-chain, Light chain; LD_{50}, Lethal dose for 50% of test animals; NAP, Neurotoxin-associated protein; PBS, Phosphate-buffered saline; SV2C, Synaptic vesicle glycoprotein 2 isoform C; TCEP, Tris(2-carboxyethyl)phosphine; TeNT, Tetanus neurotoxin; TMAO, Trimethylamine N-oxide.

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the European Pharmacopoeia describes a toxicity test in mice, which measures the toxin dose that is lethal for 50% of the animals (LD_{50}). Alternative methods for the *ex vivo* or *in vitro* measurement of BoNT activity may also be used, provided that they have been validated against the mouse LD_{50} test (Council of Europe, 2017a, 2017b). Two alternative assays have already gained approval for BoNT potency measurements from the regulatory authorities in Europe and in the USA (Fernández-Salas et al., 2012; Merz Pharma, 2015). But as these methods are product-specific and patented, and therefore not freely available to all potential users, the LD_{50} test is still widely used. It has been estimated that > 600,000 mice are used worldwide each year for the potency determination of BoNT products (Bitz, 2010), with 48,000 mice used in 2015 in Germany alone (German Federal Institute for Risk Assessment, 2018). Since the demand for BoNT products is steadily growing, there is a strong need for universally applicable and freely available *in vitro* methods suitable for replacing the LD_{50} test for BoNT potency testing.

BoNT molecules have a sophisticated mode of action. They consist of a heavy chain (H-chain) and a light chain (L-chain) interconnected by a disulfide bridge. These BoNT molecules form complexes with several neurotoxin-associated proteins (NAPs), which support the toxin during its gastrointestinal passage (Gu and Jin, 2013). Upon intestinal uptake, the complexes disintegrate, and the BoNT molecules enter the bloodstream. The BoNT serotype A recognizes two different types of receptors on motor neurons: the synaptic vesicle glycoprotein SV2 (Dong et al., 2006) and ganglioside GT1b (P1 et al., 2008). After receptor binding, the toxin is endocytosed, and its H-chain forms a transmembrane pore through which the L-chain is translocated into the cytoplasm (Koriazova and Montal, 2003). The cleavage of the interchain disulfide bond then results in the cytosolic release of the L-chain and in the activation of its proteolytic domain. The activated L-chain finally cleaves proteins involved in the exocytosis of neurotransmitters. For BoNT/A, the specific substrate is SNAP-25 (Blasi et al., 1993; Schiavo et al., 1993), a protein required for the fusion of acetylcholine-containing synaptic vesicles with the cell membrane. By inhibiting neurotransmitter release from motor neurons, BoNTs induce a paralysis of the corresponding muscles.

Here we present a biochemical assay that allows the reliable *in vitro* detection of active BoNT/A by mimicking the toxin's natural mode of action. This method is based on the binding and cleavage (BINACLE) assay strategy that allows activity measurements of clostridial neurotoxins by taking into account their two most important functions: (1.) their ability to bind to specific receptors, and (2.) their specific proteolytic activity. This assay strategy was originally developed for the sensitive *in vitro* detection of residual amounts of active tetanus neurotoxin (TeNT) during the production of tetanus toxoid vaccines (Behrensdorf-Nicol et al., 2013; Behrensdorf-Nicol et al., 2010). In addition, the applicability of the BINACLE assay principle to activity measurements of the BoNT serotype B has been demonstrated recently (Wild et al., 2016).

2. Material and methods

2.1. Reagents

Purified BoNT/A (2.6–2.7 × 10^8 mouse LD_{50}/mg, molecular weight 150 kDa) and BoNT/A with associated complex proteins (3.6 × 10^7 LD_{50}/mg, molecular weight 900 kDa) for research use were obtained from Metabiologics Inc. (Madison, WI, USA). Where indicated, a recombinant BoNT/A1 preparation (150 kDa, 1.4 × 10^8 mouse LD_{50}/mg) purchased from Toxogen GmbH (Hannover, Germany) was used instead. This toxin preparation has a well-characterized activity profile (Weisemann et al., 2015). The recombinant BoNT/A L-chain comprising amino acids 1 to 429 of full-length BoNT/A and the receptor-binding domain of the BoNT/A H-chain (HCcA) comprising amino acids 872 to 1296 were obtained from List Biological Laboratories (Campbell, CA, USA). The pharmaceutical BoNT/A products Xeomin® (Merz Pharma GmbH & Co. KGaA, Frankfurt, Germany), Dysport® (Ipsen Biopharm Ltd., Wrexham, UK) and Botox® (Allergan Pharmaceuticals, Westport, Ireland) were purchased *via* a pharmacy and reconstituted according to the manufacturers' recommendations. Pure BoNT/B was obtained from Metabiologics Inc., and pure TeNT from Sigma-Aldrich (Taufkirchen, Germany).

The recombinant SV2C receptor peptide containing the fourth luminal domain of human SV2 isoform C (amino acids 454–579) coupled to glutathione-S-transferase was purchased from Toxogen GmbH or from List Biological Laboratories. The peptide acetyl-GESQEDMFAKLKEKFFNEINKC, which is derived from synaptotagmin and acts as a specific receptor peptide for BoNT/B (Dong et al., 2003) was synthesized by GeneCust (Dudelange, Luxembourg). Recombinant SNAP-25 was purchased from List Biological Laboratories.

Contract manufacturing of the monospecific polyclonal rabbit antibody specifically recognizing cleaved SNAP-25 was performed by Biotrend Chemikalien GmbH (Cologne, Germany). For this, rabbits were immunized by intramuscular injection with the peptide CRIDEANQ, which was coupled to *Limulus polyphemus* hemocyanin and mixed with an adjuvant containing lipopolysaccharides of *Phormidium spec.* (Biotrend Chemikalien GmbH). This immunization peptide represents the amino acids 191–197 of SNAP-25 with a cysteine added for the coupling reaction. Following the primary immunization on day 0, booster injections were performed on days 7, 14, 28, 56, and 63. Sera were collected on days 63 and 70, and the cleavage site specific antibodies were affinity-purified using the peptide CRIDEANQ coupled to a cyanogen bromide-activated sepharose matrix.

Biotinylated goat-anti-rabbit-IgG and horseradish peroxidase-conjugated streptavidin were obtained from Dianova (Hamburg, Germany). Protease-free bovine serum albumin (BSA) was ordered at Serva (Heidelberg, Germany). Trimethylamine *N*-oxide (TMAO) was obtained from Sigma-Aldrich or Tokyo Chemical Industry UK Ltd. (Oxford, United Kingdom). All other reagents were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich.

2.2. BoNT/A BINACLE assay

Unless otherwise indicated, the volumes added to each well of the microtiter plates were 100 µl for incubation and coating steps, 250 µl for blocking steps, and 300 µl for washing steps.

A receptor plate was prepared by coating a transparent 96 well MaxiSorp microtiter plate (Thermo Fisher, Braunschweig, Germany) with 5 µg/ml ganglioside GT1b and 10 to 15 µg/ml SV2C receptor peptide in phosphate-buffered saline (PBS), pH 7.1 for 2 h at 37 °C and 250 rpm in a microplate thermoshaker. Wells coated only with PBS were used as controls for nonspecific binding. After coating, the plate was washed four times with washing buffer (PBS, 0.05% Tween 20) and blocked with PBS, 1% BSA for 2 h at 37 °C and 250 rpm. In the meantime, the test samples were prepared by diluting BoNT/A (or, where indicated, other toxins or toxin fragments) in 9.6 mM phosphate buffer (salt-free, pH 7.1) with 1% BSA to the final concentrations indicated in the Results section. The receptor plate was then washed four times with washing buffer, and the sample solutions were added and incubated at 4 °C overnight to enable the toxin to bind to the receptor molecules.

In parallel to the binding step, another MaxiSorp microtiter plate was coated with 100 nM SNAP-25 in PBS for 2 h at 37 °C and 250 rpm, and then blocked with PBS, 5% sucrose, 0.5% BSA, 100 µg/ml asolectin overnight at 4 °C.

On the next day, the receptor plate containing the bound toxin was washed four times using 9.6 mM salt-free phosphate buffer pH 7.1 with 0.05% Tween 20, and once using phosphate buffer without Tween. Then a reduction buffer [9.6 mM phosphate buffer pH 7.1, 5% sucrose, 2,5 mM Tris(2-carboxyethyl)phosphine (TCEP)] was added and incubated for 30 min at 37 °C and 250 rpm in order to separate the L-chains from the receptor-bound H-chains. In the meantime, the SNAP-