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Inhibiting oral intoxication of botulinum neurotoxin A complex by carbohydrate receptor mimics

Kwangkook Lee [a, 1], Kwok-Ho Lam [a, 1], Anna-Magdalena Kruel [b], Stefan Mahrhold [b], Kay Perry [c], Luisa W. Cheng [d], Andreas Rummel [b, *], Rongsheng Jin [a, *]

[a] Department of Physiology and Biophysics, University of California, Irvine, CA 92697, USA
[b] Institut für Toxikologie, Medizinische Hochschule Hannover, Hannover, Germany
[c] NE-CAT and Department of Chemistry and Chemical Biology, Cornell University, Building 436E, Argonne National Laboratory, 9700 S. Cass Avenue, Argonne, IL 60439, USA
[d] Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, United States Department of Agriculture, Agricultural Research Service, Albany, CA 94710, USA

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ABSTRACT

Botulinum neurotoxins (BoNTs) cause the disease botulism manifested by flaccid paralysis that could be fatal to humans and animals. Oral ingestion of the toxin with contaminated food is one of the most common routes for botulism. BoNT assembles with several auxiliary proteins to survive in the gastrointestinal tract and is subsequently transported through the intestinal epithelium into the general circulation. Several hemagglutinin proteins form a multi-protein complex (HA complex) that recognizes host glycans on the intestinal epithelial cell surface to facilitate BoNT absorption. Blocking carbohydrate binding to the HA complex could significantly inhibit the oral toxicity of BoNT. Here, we identify lactulose, a galactose-containing non-digestible sugar commonly used to treat constipation, as a prototype inhibitor against oral BoNT/A intoxication. As revealed by a crystal structure, lactulose binds to the HA complex at the same site where the host galactose-containing carbohydrate receptors bind. *In vitro* assays using intestinal Caco-2 cells demonstrated that lactulose inhibits HA from compromising the integrity of the epithelial cell monolayers and blocks the internalization of HA. Furthermore, co-administration of lactulose significantly protected mice against BoNT/A oral intoxication *in vivo*. Taken together, these data encourage the development of carbohydrate receptor mimics as a therapeutic intervention to prevent BoNT oral intoxication.

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1. Introduction

Botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* are the most poisonous toxins, and classified by the Centers for Disease Control and Prevention as one of the six highest-risk threat agents for bioterrorism (Arnon et al., 2001). They specifically cleave the soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) after invading motoneurons at neuromuscular junctions and subsequently block acetylcholine release (Rossetto et al., 2014). Current treatment for botulism requires early diagnosis, immediate treatment with equine antitoxin, prolonged hospitalization in an intensive care unit and mechanical ventilation (Rusnak and Smith, 2009). Hence, there is an urgent need to develop effective diagnostic and preventive countermeasures against oral BoNT intoxication.

There are seven serotypes of BoNT (termed BoNT/A–G) including at least 40 different subtypes, among which BoNT/A, B, E, and F are known to cause human botulism (Rossetto et al., 2014). BoNTs are naturally secreted in the form of progenitor toxin complex (PTC) where the toxin is bound to several non-toxic neurotoxin-associated proteins (NAPs). NAPs are encoded together with the toxin gene in one of two different gene clusters, the *HA* cluster or the *orfX* cluster (Hill and Smith, 2013). Besides a common non-toxic non-hemagglutinin (NTNHA) protein, the *HA* gene cluster (BoNT/A1–D and G) encodes three hemagglutinins (HA70, HA17, and HA33) and the *orfX* cluster (BoNT/A2–4, E, F) encodes three proteins termed orfX1-3 (Gu and Jin, 2013; Kubota et al., 1998; Lam

Abbreviations: BoNT, Botulinum neurotoxin; LAU, Lactulose; HA, Hemagglutinin; PTC, Progenitor toxin complex; NTNHA, Non-toxic non-hemagglutinin.

* Corresponding authors.
E-mail addresses: rummel.andreas@mh-hannover.de (A. Rummel), r.jin@uci.edu (R. Jin).

[1] Authors contributed equally to this work.

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and Jin, 2015). The function of the orfX proteins remains mysterious, so they will not be included in the following discussion. NAPs are crucial for the delivery of BoNTs across the epithelial barrier into systemic circulation, as BoNTs themselves are sensitive to inactivation and degradation in the hostile environment of the gastrointestinal (GI) tract (Gu et al., 2012; Shone et al., 1985). The oral toxicity of BoNTs is increased by hundreds to thousands folds in the form of PTC compared to the naked toxin (Ohishi, 1984; Ohishi et al., 1977).

The large PTC (L-PTC) contains two structurally and functionally distinct modules. BoNT and NTNHA form an oval-shaped interlocked complex (M-PTC), which protects the toxin against digestive enzymes and the acidic environment in the gut (Gu et al., 2012). The three HAs form an extended three-blade architecture (HA complex) that is composed of HA70, HA17 and HA33 in 3:3:6 stoichiometry (Amatsu et al., 2013; Benefield et al., 2013; Lee et al., 2013). HA70 and HA33 of L-PTC/A and B carry an N-acetylneuraminic acid (Neu5Ac) and a galactose (Gal) binding site, respectively. Therefore, each HA complex of L-PTC/A and B comprises a total of nine glycan-binding sites, which allow multivalent interactions with host carbohydrates to enrich the toxin complex on the intestinal surface (Lee et al., 2013; Matsumura et al., 2015; Sugawara et al., 2014; Yao et al., 2014). The HA complex then interacts with E-cadherin, a major host adhesion protein, to disrupt the E-cadherin mediated cell–cell adhesion and open the paracellular route facilitating the transepithelial delivery of BoNT (Lee et al., 2014b; Matsumura et al., 2008; Sugawara et al., 2010). Interestingly, a recent study suggests that the carbohydrate-binding activity of HA may help the L-PTC to exploit microfold (M) cells to breach the intestinal epithelial barrier, which is mediated by glycoprotein 2 (GP2) on the cell surface (Matsumura et al., 2015).

The substantial recent advances in understanding the structure and function of HA-carbohydrate interactions suggest a new strategy for the development of preventive countermeasures for BoNTs based on carbohydrate receptor mimicry. For instance, isopropyl β-D-1-thiogalactopyranoside (IPTG), a non-metabolizable Gal analog, could inhibit the oral toxicity of L-PTC/A using a mouse model (Lee et al., 2013). In this study, we have identified lactulose (LAU), 4-O-β-D-galactopyranosyl-D-fructose, as another potential inhibitor. We have performed thermodynamic analysis on binding between HA33 and LAU, and resolved the crystal structure of their complex. The physiological relevance of the HA33-LAU interaction was further examined by an *in vitro* model using Caco-2 epithelial cells and by an *in vivo* mouse oral toxicity assay.

2. Material and methods

2.1. Construct design and cloning

The constructs of HA70, HA17, and HA33 were prepared as previously reported (Lee et al., 2013). Briefly, full length HA17 (residues M1–I146) and full length HA33 (residues M1–P293) from *C. botulinum* BoNT/A1 were cloned separately into the bicistronic pRSFDuet-1 vector for co-expression. HA17 was produced with an N-terminal 6xHis tag to facilitate protein purification while HA33 carries no tag. Full length HA70 (residues M1–N626) was cloned into the expression vector pQE30.

2.2. Protein expression and purification

Protein expression and purification were performed as described previously (Lee et al., 2013). Briefly, HA17 and HA33 were co-expressed in *Escherichia coli* strain BL21-RIL (DE3) (Novagen). The HA17–HA33 complex was first purified by Ni-NTA (nitrilotriacetic acid, Qiagen) affinity column. His-tag was removed by PreScission protease and the protein was further purified by MonoS ion-exchange chromatography and Superdex 200 size-exclusion chromatography. The protein complex was concentrated to ~6 mg/ml using Amicon Ultra centrifugal filter (Millipore) for crystallization. The complete HA complex was reconstituted by mixing HA70 and the HA17–HA33 complex at a molar ratio of ~1:1.3 and the complex was further purified by Superdex 200 chromatography. The fluorescence-labeled HA complex (HA*) was prepared with Alexa Fluor® 488 labeled HA70 and unlabeled HA17–HA33 complex (Lee et al., 2013).

2.3. Isothermal titration calorimetry

The calorimetry titration experiments were performed on an ITC200 calorimeter from Microcal/GE Life Sciences (Northampton, MA). LAU (40 mM) was used as the titrant in the syringe and HA33 (200 μM) was used as the titrand in the cell. The data were analyzed using the Origin software package. The thermodynamic values reported are the mean of three independent experiments.

2.4. Crystallization, data collection and structure determination

Crystals of the HA17–HA33 complex were prepared as described previously (Lee et al., 2013). Protein-carbohydrate complexes were obtained by soaking the HA17–HA33 crystals with 100 mM IPTG or LAU at 18 °C overnight. X-ray diffraction data were collected at the Advanced Photon Source (APS). The crystals belong to space group $C222_1$, with unit cell dimensions $a = 107$ Å, $b = 119$ Å, $c = 162$ Å; $\alpha = \beta = \gamma = 90°$. The data were processed with HKL2000 (Otwinowski and Minor, 1997). Data collection statistics are summarized in Table 1. The structures were determined by molecular replacement software Phaser using apo-HA17–HA33 (PDB code 4LO0) as the search model (Lee et al., 2013; McCoy et al., 2007). Manual model building and refinements were performed using COOT, PHENIX, and CCP4 packages in an iterative manner (Adams et al., 2010; Emsley et al., 2010; Winn et al., 2011). IPTG and LAU were modeled into the corresponding structure during the refinement based on the Fo-Fc electron density maps. The refinement progress was monitored with the free R value using a 5% randomly selected test set. The structures showed excellent stereochemistry based on MolProbity validation (Chen et al., 2010). Structural refinement statistics are listed in Table 1. All structure figures were prepared with PyMol. (http://www.pymol.org).

2.5. Transwell assay

The transwell assay was performed as previously described (Lee et al., 2013, 2014b). Caco-2 cells were obtained from the German Cancer Research Center (Heidelberg, Germany). The cells were subcultured twice a week and seeded on BD Falcon Cell Culture Inserts (0.9 cm^2) at a density of approximately 10^5 cells cm^{-2} for flux studies and determination of transepithelial electrical resistance (TER). All TER experiments were conducted in 0.5 ml and 1.5 ml of Iscoves Modified Dulbeccos Medium without phenol red in the apical and basolateral reservoir, respectively. LAU (95%, Sigma–Aldrich, Taufkirchen, Germany) was dissolved in IMDM, sterile filtered and stored at −20 °C. The HA* was pre-incubated with LAU overnight at 4 °C in IMDM and diluted to the final concentration with IMDM prior to administration. The TER upon administration of LAU in the highest concentrations used was checked in the absence of HA* and was virtually identical to that of the control without sugars. The HA* was administered to the apical or basolateral reservoirs at final concentrations of 58 nM and 17 nM, respectively. TER was determined with an epithelial volt–ohm meter (World

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