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Production and characterization of a neutralizing antibody against botulinum neurotoxin A

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Abstract

As a category A toxic, the botulinum toxin(BoNT) is responsible for human botulism with an estimated lethal dose of 1 ng/kg which greatly increases the potential risk of use as bioweapons. Therefore, the development of anti-BoNT antibodies is urgent. In this paper, the H_C domain of BoNT/A was purified and immunized with Balb/c mice. Monoclonal antibodies were screened against BoNT/A from 55 stable positive hybridoma cell lines, and one with the strongest neutralizing activity, designated as ML06, was subcloned, sequenced, and classified as IgG1(κ) subclass. The mouse protection assays showed that ML06 can neutralize the toxin of BoNT/A effectively both *in vitro* and *in vivo*, in a dose-dependent manner. The therapeutic assays showed that only 20% of mice injected with 4 LD_{50} BoNT/A can survive another injection of ML06 after 4 h. The prophylaxis assays showed the residual ML06 from mice injected with ML06 two weeks ago can protect mice against 4 LD_{50} BoNT/A challenge completely. Collectively, our results indicated that ML06 served as a good candidate for further development of immune therapeutics for BoNT/A.

Keywords: MLD_{50}; botulinum neurotoxin; monoclonal antibody; mouse neutralization assay; prophylaxis assays

1 Introduction

Botulinum neurotoxin (BoNT) is a toxic protein secreted by bacterium *Clostridium botulinum* and related *Clostridium* species during the spore-forming. Seven serotypes (A, B, C, D, E, F, G) have been found in the past 45 years [1], and a new serotype H has been identified recently [2]. Serotype A, B, E, and rarely F can cause botulism disease in humans, manifested as flaccid muscle paralysis, dizziness, and expiratory dyspnea [3]. Foodborne botulism, wound botulism and infant botulism are three common forms of botulism caused by BoNT. Foodborne botulism is either food poisoning of BoNT or the *Clostridium botulinum* colonization of the gastrointestinal tract [4].

Botulinum neurotoxin A (BoNT/A) is the most toxic substance among the eight serotypes. Extrapolated from the data of non-human primates, the 50% lethal dose values (LD_{50}) of human beings was 3 ng/kg by the pulmonary aspiration and 1 ng/kg by the intravenous and subcutaneous injection. Therefore, BoNT/A was classified as category A toxic and posed a major bioweapon threat [5].

The primary structure of BoNT/A contains two peptide chains: the light chain and the heavy chain which are linked by a disulfide bond. Both the light and heavy chains are hydrolyzed by a proteolytic enzyme from a 150 kDa single-chain protoxin. The light chain contains 437 amino acids forming a zinc endopeptidase which can hydrolyze synaptosomal associated protein (SNAP25) at the site of amino acid 197. The digested SNAP25 inhibits the fusion of presynaptic membrane with synaptic vesicle, and then blocks acetylcholine release, causing flaccid paralysis [6]. The heavy chain contains 848 amino acids forming two functional domains (H_C and H_N). The H_C domain mediates the specific binding of BoNT/A to polysialoganglioside (PSG) and the luminal domain of synaptic vesicles (SV) protein of presynaptic membrane, which leads to the internalization of BoNT/A into nerve cells [7]. The H_N domain is responsible for the translocation of the light chain of BoNT/A from synaptic endosome interior into the cytosol [8].

Although BoNTs is lethal, the case of botulism caused by BoNTs is rare in our life. BoNTs were extensively used for medical purposes, such as smooth muscle hyperactivity treatment, glandular hypersecretion treatment, and chronic pain-associated conditions treatment [9]. Therefore, military or universal vaccination is unlikely, and no FDA approved vaccine for botulism was used for human beings at present [10]. Currently the main way to treat botulism is passive immunization with human anti-BoNT immunoglobulins, such as BabyBIG [11] and heptavalent equine anti-BoNT serum [12]. However, hypersensitivity and serum sickness reaction could be caused by the equine serum, and the stock of the human serum of BabyBIG is rather limited.

Therefore, to develop monoclonal antibodies (McAbs) against BoNTs was the best strategy for botulism treatment, which can be produced at high quality and on a large scale. Some mouse monoclonal antibodies that showed neutralizing activities against BoNT/A have been established using the C-terminal of BoNT/A [13]. Recent work described human McAbs binds the light chain of BoNT/A [14]. However, single McAbs can neutralize at most 10 to 100 times of the LD_{50} of toxin in mice, and a combination of different McAb is required for efficient neutralization. In the present study, we aimed to produce a specific monoclonal antibody against BoNT/A and evaluate its ability to protect against BoNT/A toxicity *in vitro* and *in vivo*, and determined the antibody subtype, variable

region sequence, affinity, and binding subdomain. This McAb serves as a good candidate for further development of immune therapeutics for botulism caused by BoNT/A.

2 Materials and Methods

2.1 Ethics statement

This study was accomplished by the recommendations in the Guide for the Care and Use of Laboratory Animals of the Academy of Military Medical Sciences Ethics Committee. The experiments and protocol were approved by the Committee on the Ethics of Animal Experiments of the Academy of Military Medical Sciences (ID: SYXK2014-06). All animal experiments were performed under isoflurane anesthesia and conform to the relevant regulatory standards to minimize animal suffering.

2.2 Chemicals, reagents, cell lines, and mouse

BoNT/AH_C antigen was produced and stored in our lab. Myeloma cells SP2/0 were stored in our lab. Freund's complete and incomplete adjuvant and mercaptoethanol were obtained from Sigma Chemical Co. Hypoxanthine, thymidine (HAT), thymidine (HT), aminopterin, and hypoxanthine were obtained from Thermo Fisher Co. HRP conjugated anti-mouse IgG was obtained from Thermo Fisher Co. Penicillin, streptomycin, fetal bovine serum (FBS), and DMEM were purchased from Gibco. Ni-NTA resin, Protein G sepharose column, and CM Sepharose Fast Flow were obtained from GE healthcare. Mouse monoclonal antibody typing reagent (IgG1, IgG1a, IgG2b, IgG3, IgM, IgA, Kappa, lamda) were obtained from BioDragon Co.

The murine myeloma SP2/0 cells and hybridoma cells secreting mouse ML06 McAbs were propagated and maintained in DMEM supplemented with 10% FBS and antibiotics, at 37℃ in the presence of 5% CO2.

18–22 g male KM mouse and 8 weeks old female Balb/c mice were obtained from the Animal Center, Academy of Military Medical Sciences, and were fed ad libitum with commercial pellets. All animals were placed in socially-enriched cages (ten animals per cage) with a relative humidity of 40%–70%, and the temperature of 25±2℃.

2.3 Cloning, expression, and purification

Genes encoding the N-terminal (BoNT/AH_{CN}: 865-1097 amino acid), the C-terminal (BoNT/AH_{CC}: 1098-1296aa) of the BoNT/AH_C domain (strain 62A: 865-1296aa, accession no. M30196), and the full BoNT/AH_C length were amplified and cloned into pET22b after digesting with *Nde*I and *Xho*I. The recombinant plasmids pET22b-BoNT/AH_{CN}, pET22b-BoNT/AH_{CC}, and pET22b-BoNT/AH_C were then transferred into *Escherichia coli* BL21(DE3) respectively. The overexpression of those proteins were conducted at 20℃ for 12 h, with 0.5 mM final concentration of IPTG. The cells were centrifuged at 5000 rpm for 15 min at room temperature, and then resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl, and and a protease inhibitor cocktail).

The cell suspensions were lysed by sonication for 5 min (3 s sonication and 6 s intervals). All of the BoNT/AH_{CN}, BoNT/AH_{CC}, and BoNT/AH_C lysates were clarified by centrifugation (25,000g×40 min, 4℃), and then the supernatant was added with imidazole to 10 mM final concentration. The recombinant proteins were purified by a Ni-NTA resin column with elution buffer (lysis buffer containing 100 mM imidazole), concentrated by an Amicon Ultra filter, and dialyzed extensively against 25 mM PBS buffer, pH 6.8. The recombinant proteins were then applied to a cation-exchange column for further purification. The BoNT/AH_{CN} and BoNT/AH_{CC} were eluted with PBS containing 200 mM NaCl, and the BoNT/AH_C were eluted with PBS containing 250 mM NaCl. And then, the purified proteins were pooled and dialyzed against 50 mM PBS, pH 7.4. The purity of those three polypeptides were checked by 10% SDS-PAGE, and concentrated to 2 mg/ml for further experiments.

2.4 Generation of hybridomas and collection of mouse ascites

Immunization of Balb/c mice (8 weeks old, female) was conducted by subcutaneous injections of 50 μg purified BoNT/AH_C emulsified with Freund's complete adjuvant. After 3 weeks, those mice were further immunized by subcutaneous injections with 100 μg Freund's incomplete adjuvant BoNT/AH_C twice 3 weeks apart. Mice with high anti-BoNT/AH_C IgG titers determined by ELISA were selected, and those mice were administrated i.p. with 100 μg of BoNT/AH_C diluted in PBS. Three days after the last injections, selected mice were sacrificed and their spleens were aseptically collected.

Mice spleen cells and myeloma cells SP2/0 were fused in the ration of 5 to 1 using PEG1500. The fused cells were plated in DMEM supplemented with 20% (*v/v*) FBS, 40 mM aminopterin, 10 mM hypoxanthine, and 1.6 mM thymidine. They were examined for the secretion of anti-BoNT/AH_C-specific antibodies using ELISA after 10 days. The positive clones were subcloned to monoclonality by two rounds of limiting dilution subcloning. The specific immunoglobulin class of each McAb was determined using a mouse isotyping kit. The titer of the anti-serum and cell culture supernatant was determined by indirect ELISA.

To obtain large numbers of McAbs, 1.0×10^6 hybridoma cells were injected i.p. into an eight weeks old Balb/c mouse which was injected with 0.5 ml atoleine a week previously. When the abdomen of the mouse was swollen for 7 days, the ascites were collected. McAbs were purified by Protein-G Sepharose affinity chromatography according to the manufacture's instruction (GE). The concentration of McAbs was checked by BCA reagent assay kit.

2.5 ELISA on recombinant BoNT/AH_C protein

ELISA was conducted in microtiter plates. Plates were coated with 100 μl carbonate buffers (50 mM, pH 9.6) containing 100 ng BoNT/AH_C at each well for 12 h at 4℃. After three PBST (300ul PBS, 0.05% Tween 20) washes, the plates were blocked with 300 μl PBST containing 5% skim milk for 1.5 h. Subsequently, the McAbs were diluted in PBST containing 2% skim milk and incubated in the BoNT/AH_C-coated wells for 1.5 h. After three PBST washes, a HRP conjugated anti-mouse antibody diluted in PBST containing 2% skim milk was incubated in the BoNT/AH_C-coated wells for 1 h. After three PBST washes, the bound anti-mouse antibody was determined with the staining reaction and the absorbance was measured at 450 nm in a BioRad microtiter plate reader.

2.6 Cloning and sequencing of Ig V_H and V_L genes

Total RNA of BAS45 hybridoma cells that produce an antibody against BoNT/AH_C was extracted and cDNA was synthesized using Trizol and cDNA reverse transcription kit (Invitrogen, USA). Both the Ig V_H and V_L genes were amplified by PCR reactions using cDNA as a template. The V_H region was amplified using GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT as the 5' primer and GACHGATGGGGSTGTYGTGCTAGCTGMRGGACDGTGA as the 3' primer. The V_L region was amplified using GGGGATATCCACCATGGAGACAGACACACTCCTGCT AT as the 5' primer and GGATACAGTTGGTGCAGTCGACTTACGTTTKATTTCCARCTT as the 3' primer. The PCR products were extracted and cloned into vector pMD18T by T4 ligase. After screening by colony PCR, the positive clones were sequenced from both directions using M13F and M13R primers and subjected to IMGT/V to determine the V and J region of IgG, respectively.

2.7 Affinity and cross-reactivity measurement

The affinity of ML06 McAbs to BoNT/AH_C was determined by a surface plasmon resonance instrument (General Electric-Biacore). ML06 McAbs was captured on a biosensor chip. The purified BoNT/AH_C was extensively diluted in 25 mM HEPES buffer containing 0.01% Tween as previously described [15, 16]. The experiments were performed for 5 cycles, with 10 nM, 20 nM, 40 nM, 80 nM, and 160 nM BoNT/AH_C. The K_D values were calculated from the ratio of K_{off} and K_{on}. To test the specificity of ML06 McAb, western blotting was performed as described before [17].

2.8 Neutralization assay *in vitro* and *in vivo*

To assess the neutralizing activity of ML06 McAb *in vitro*, we performed a mouse bioassay. The BoNT/A protein solution was prepared in PBS, to 4 times of murine lethal doses (MLD_{50}). The diluted BoNT/A was incubated with 0, 2, 4, 8, and 16 μg ML06 McAb for 30 min. 500 μl of the incubated BoNT/A and ML06 McAb mixture was injected i.p into male KM mice (18–22 g, n=5 per group). Mice were monitored daily for mortality up to 4 days post-infection.

To assess the neutralizing activity of ML06 McAb *in vivo*, the mouse was injected with both ML06 McAb and BoNT/A without pre-incubation. 500 μl 4 MLD_{50} of BoNT/A in PBS were injected i.p. into KM mice (18-22 g, n=5 per group). Concomitantly, 500 μl 0, 2, 4, 8, and 16 μg ML06 McAb in PBS was injected i.v. into mice at a close but different site. Mice were monitored daily for mortality up to 4 days post-infection.

2.9 Influence of the interval between BoNT/A and ML06 McAb injections

500μl 4 MLD_{50} of BoNT/A were injected i.v. into seven groups KM mice (18-22 g, n=5 per group), and then 500 μL ML06 McAb were injected i.p. after 0 h, 0.5 h, 1 h, 2 h, 4 h, or 6 h. The concentration of ML06 McAb injected in this experiment was equivalent to 10 times the dose which can protect the mice challenged with 4 MLD_{50} of BoNT/A in neutralization assay *in vivo*. Mice were monitored daily for mortality up to 4 days post-infection.

2.10 Prophylaxis Assay

To test the prophylactic capacity of ML06 McAb, 100 μg of ML06 McAb was injected i.p. into five male KM mice (18-22 g). And then, 500 μl 4 MLD_{50} of BoNT/A was administered i.v. into each mouse at 1, 3, 7, and 14 d after ML06 injection. Mice were observed and survival was assessed for 4 days after the last injection.

3 Results

3.1 Immunization with purified BoNT/AH_C

The recombinant BoNT/AH_C, plus a C terminal His_6-tag, had been expressed in *E. coli* BL21(DE3) and sequentially purified by Ni-NTA and anion-exchange column respectively (**Fig. 1A)**. A clear band corresponding to BoNT/AH_C was detected by 10% SDS-PAGE, which indicates the purity of BoNT/AH_C was greater than 95% (**Fig. 1B)**.

Immunization of mice with purified BoNT/AH_C protein resulted in the development of humoral immune responses. After four cycles of immunization, mouse sera were subjected to ELISA assay with 1 μg BoNT/AH_C coated plates. Results showed that the maximum titer of all mice sera reached 1:32,000, and the sera of mouse #5 contained the highest titers of antibodies against BoNT/AH_C (**Fig. 1C**). Therefore, mouse #5 was selected to accept another impact immunization, and its spleen cells were aseptically collected.

3.2 Screening of hybridoma against BoNT/AH_C

The fusion of spleen cells obtained from mouse #5 with myeloma cells SP2/0 enabled the generation of anti-BoNT/AH_C hybridoma cell lines. After two weeks, the fusion rate of hybridoma cells was calculated by the microscopic method and the cell lines that produce IgG specific for BoNT/AH_C were screened by ELISA. 357 hybridoma cell lines from 4 plates (96-well) were screened, and the total fusion rate was up to 92.96% (**Table 1)**. Of those cell lines, 97 hybridoma cell lines producing anti-BoNT/AH_C antibody were screened by ELISA.

The positive cell lines were subcloned by two rounds of limiting dilution and finally obtained 55 stable hybridoma clones, named BAS1-BAS55. After expanding culture, the titer of cell culture supernatant was determined by indirect ELISA and the subclass was analyzed by mouse monoclonal antibody isotype assay kit. Results showed that the titer of hybridoma clones supernatant ranged from 10^2 to 10^4, as listed in **Table 2**. 24 McAbs were of the IgG1(κ), 3 of the IgG2a(κ), 14 of the IgG2b(κ), 1 of the IgM(κ) subclass, and 5 McAbs with lower titers can't be classified.

To select McAb with the highest protective capacity against BoNT/A, 400 μl of each hybridoma clone supernatant was incubated with 4 MLD_{50} BoNT/A for 30 min at room temperature and injected i.p. into KM mice for protection assays. Finally, BAS45 hybridoma clone supernatant exhibited the highest protective capacity, and the McAb was designated as ML06.

3.3 Sequence, specificity, and epitope characterization of ML06 McAb

The cDNA encoding ML06 McAb was isolated by RT-PCR from hybridoma clone BAS45. The amplified cDNA was sequenced and translated into amino acid (showed in CP201910584470.X), characterized as IgG1(κ) subclass. After the sequencing of ML06 McAb, we performed a blast analysis with the mouse germline gene using the IMGT/V-QUEST tool. The results showed that the variable regions in IgG (V_H) and (V_L) of ML06 shared 93.68% and 96.42% identity to the original sequences of V gene, suggesting that V_H and V_L of ML06 remained less mutated after the maturation. The identified V gene segments of V_H and V_L, CDR length, and CDR3 sequence in its heavy chain and light chain were all listed in **Table 3**.

To obtain sufficient amounts of ML06 McAb, we injected the hybridoma cells BAS45 into Balb/c mice intraperitoneally. The ascites was collected and subjected to purification by Protein-G affinity chromatography. SDS-PAGE was used to analyze the McAb, and the result showed that the purified ML06 McAb has a band of heavy chain at 50 kDa and a band of light chain at 27 kDa (**Fig. 2A**).

Since BoNT/A shared a high similarity of protein sequences with BoNT/B, BoNT/E, and BoNT/F, we wonder whether ML06 contains any cross-reaction among different serotypes of BoNTs. Western blot analysis showed that ML06 only recognized BoNT/A and BoNT/AH_C, but failed to bind BoNT/B, E, and F (**Fig. 2B**). These results suggest that ML06 bound BoNT/A by specifically recognizing the BoNT/AH_C domain without any cross-reaction to the other three serotypes. The affinity of ML06 and BoNT/AH_C was determined with Biacore at different antigen concentrations, and the affinity was in the subnanomolar range (1.16×10^{-8}mol/L).

To further map the epitope of ML06, two truncated fragments (BoNT/AH_{CN} and BoNT/AH_{CC}) of BoNT/AH_C were cloned, expressed, and purified. SDS-PAGE analysis showed that the observed molecular mass of H_{CN} was ~25kDa and H_{CC} was ~22kDa, which was consistent with the calculated molecular weight, and the purity was greater than 95% (**Fig. 3A**). Western blot analysis showed that ML06 only recognized BoNT/AH_{CC} but BoNT/AH_{CN} (**Fig. 3B**). These results suggested that ML06 bound BoNT/A by specifically recognizing a linear epitope of H_{CC} domain of BoNT/AH_C subunit, other than a conformational epitope.

3.4 Neutralization assay with ML06 McAb *in vitro* and *in vivo*

To assess the *in vitro* neutralizing activity of ML06 McAb, mouse protection assay was conducted by injection i.p. with BoNT/A (4 MLD_{50}) pre-incubated with ML06. Results showed that the highest survival rate (100%) was the mice group injected with 16μg/ml ML06, while the survival rate of mice group injected with 8 and 4 μg/ml ML06 was 60% and 20%. And the mice groups injected with less than 2 μg/ml ML06 all succumbed to.botulism, as shown in **Fig 4A**. 16 μg of ML06 McAb can completely protect mice against a 4 MLD_{50} challenge of BoNT/A, emphasized the great potency of ML06 McAb in neutralizing BoNT/A toxicity in mice.

Moreover, the protective activity of ML06 McAb was tested *in vivo* by the injecting BoNT/A and ML06 separately, without mixing or *in vitro* pre-incubation. As shown in **Fig. 4B**, 10 μg of ML06 McAb by i.p. can protect all the mice challenged with 4 MLD_{50} BoNT/A completely, while 8 μg of ML06 only protected the 40% mice challenged with 4 MLD_{50} BoNT/A. Altogether, ML06 McAb can neutralize the toxin of BoNT/A effectively *in vitro* and *in vivo*, and the neutralizing activity was increased with the ML06 dose.

3.5 Influence of the interval between BoNT/A and ML06 McAb injections

To determine the influence of the interval between BoNT/A and ML06 McAb injections, we assessed animal survival rate at different intervals (**Table 4)**. When 4 MLD_{50} of BoNT/A and 100 μg of ML06 McAb were injected i.p. at the same time, the mice's survival rate was 100%. It would maintain at 100% while the interval between BoNT/A and ML06 McAb injections was 0.5 h and 1 h. From 2 h onwards, the survival rate decreased as the time between BoNT/A and ML06 injections increased: 60% for 2 h, 20% for 4 h, 0% for 6 h. Since the clinical signs and symptoms of human

botulism appeared within 4h after exposure to 4 MLD_{50} of BoNT/A, it means 20% of patients could survive and recover.

3.6 Prophylaxis assays performed with ML06 McAb

The prophylactic capacity of ML06 McAb was also tested by mouse protection assay. 100 μg of ML06 McAb was injected i.p. into every mouse on day 0, 1, 3, 7, 14 before administration of BoNT/A by i.p. injection. All mice injected with ML06 McAb up to 14 days in advance survived after receiving a test dose of 4 MLD_{50} BoNT/A (**Table 4),** indicating that ML06 McAb has a good prophylactic capacity.

4 Discussion

Neutralizing antibodies recognizing BoNT have been reported previously, recognizing different epitopes on BoNT [19-21]. In this study, 55 McAbs were screened out and recognized the receptor-binding domain of BoNT/A, which was used as an antigen. Among these McAbs, ML06 had the highest neutralizing activity (250 LD_{50}/mg), which binds to BoNT/A in the affinity of 11.6 nM. Previously reported McAb had an affinity range of 1 to 100 nM, and neutralizing potency range of hundreds fold of LD50/ mg antibody [15, 16]. For ML06 McAb, its neutralizing activity and affinity were also within these ranges, and humanization and affinity maturation would be further applied to decrease immunogenicity and improve neutralizing activity in the future.

We found that ML06 McAb didn't recognize BoNT/B, BoNT/E, BoNT/F, but specific for BoNT/A. In general BoNT serotypes share similar amino acid sequences, but ML06 McAb can only bind the H_C domain of BoNT/A, perhaps due to the lack of sequence conservation within the last 200 amino acid residues of the H_C region of BoNTs [1, 22-24].

Previous studies on BoNT/A epitopes found that these epitopes are easily denatured and may be discontinuous or conformational [25-27]. The results described here show ML06 McAb binding to a linear epitope. ML06 McAb was further used to define a distinct, protective epitope on $BoNT/AH_{CC}$. Detailed characterization of its epitope and identification of its location within the $BoNT/AH_C$ sequence may help to create a more effective peptide-based vaccine.

Moreover, defining the protective epitope on $BoNT/AH_C$ antigen will increase our knowledge of the interaction between BoNT and its receptors. Two receptors were involved in the binding of the toxin RBD [28-30]. Firstly, the C-terminal portion of $BoNT/AH_C$ ($BoNT/AH_{CC}$) binds to the ganglioside GT1b, on the outer leaflet of the presynaptic membrane, albeit with low affinity [31, 32]. Secondary binding to a higher affinity protein receptor, synaptic vesicle glycoprotein 2 (SV2), adjacent to the binding site of GT1b in $BoNT/AH_{CC}$ then reinforced the interaction [31, 33]. Birgitte PS Jacky reported that FGFR3, expressed in MNTs motor neurons, was also a receptor for BoNT/A. Though the binding site for GT1b and SV2C has been identified, the binding site for FGFR3 remains unknown. SPR experiments had demonstrated that the FGFR3 and SV2C bind to sites on $BoNT/AH_C$ well apart from each other [34]. In our laboratory, the results of competitive ELISA assay show that FGFR3 binding sites overlapped with the epitope of another neutralizing monoclonal antibody (ML02), in the N-terminal portion of $BoNT/AH_C$, $BoNT/AH_{CN}$ (unpublished data).

In summary, we reported here a neutralizing McAb obtained by vaccination with the nontoxic binding fragment BoNT/AH_C. It has high affinity and recognized a neutralizing epitope, which will be proved useful for both diagnostic and therapeutic applications.

Contributions

Weicai Zhang conceived the project and designed the experiments, and modified the manuscript. Xiaodong Han analyzed the data and wrote the manuscript. Xianghua Xiong performed the expression and purification of the recombinant proteins. Sunhui Lv raised and immunized mice, and completed the neutralization assay in mice. Chuxi Fu performed the affinity experiments of antigen and antibody. Lei Li purified the antibody. Zhijie Sun analyzed the sequences of Ig V_H and V_L genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Fig.1 The expression, purification, and immunization analysis of BoNT/AH_C. (**A**) The expression of BoNT/AHc in *E. coli* was analyzed by 10% SDS-PAGE. Lane M, mid-range protein molecular weight markers. Lane CK, pTIG vector-transformed BL21(DE3) cell lysates. Lanes 1-5, pTIG-BoNT/AHc plasmid-transformed BL21(DE3) cell lysates induced with IPTG. (**B**) The purity of BoNT/AH_C was checked by SDS-PAGE. Lane M, mid-range protein molecular weight markers. Lanes 1 and 2, purified BoNT/AH_C protein. (**C**) Titers of mouse antiserum after four rounds of immunization with BoNT/AH_C.

Fig.2 Purification and specification of ML06 McAb. All proteins were analyzed by 10% SDS-PAGE and stained with Coomassie brilliant blue. (**A**) Purification of ML06 McAb. Lane M, mid-range protein molecular weight markers. (**B**) The epitope of ML06 McAb. ML06 McAb bound BoNT/A by specifically recognizing a linear epitope of Hcc domain of BoNT/AH_C subunit, other than a conformational epitope.

Fig.3 Epitope characterization of ML06 McAb. (**A**) SDS-PAGE analysis of purified BoNT/AH_{CN} and BoNT/AH_{CC} subdomain. Lane M, mid-range protein molecular weight markers. H_{CN}, the N-terminal of the BoNT/AH_C receptor-binding domain. H_{CC}, the C-terminal of the BoNT/AH_C receptor-binding domain. (**B**) Western blot analysis of purified BoNT/AH_{CN} and BoNT/AH_{CC} subdomain interacting with ML06 McAb.

Fig.4 *In vitro* and *in vivo* neutralization of botulinum neurotoxin serotype A with ML06 McAb. (**A**) *In vitro* neutralization of botulinum neurotoxin serotype A with ML06 McAb. 16 μg of ML06 McAb completely protected mice against a 4 MLD_{50} challenge of BoNT/A. (**B**) *In vivo* neutralization of botulinum neurotoxin serotype A with ML06 McAb. An intraperitoneal injection 10 μg of ML06 McAb was able to completely protect all the mice challenged with 4 MLD_{50} BoNT/A.

Table 1. Result of cell fusion and screening

No.	Fusion rate	Positive rate
1	90.63%(87/96)	13.79%(12/87)
2	93.75%(90/96)	20.00%(18/90)
3	91.67%(88/96)	9.10%(08/88)
4	95.83%(92/96)	18.48%(17/92)
Total	92.96%(357/384)	27.17%(97/357)

Table 2. The titer of hybridoma cell culture supernants and subclass of the McAbs

No.	BAS01	BAS02	BAS03	BAS04	BAS05	BAS06	BAS07	BAS08	BAS09	BAS10	BAS11
Titer	10^3	10^3	10^2	10^2	10^2	10^2	10^2	10^2	10^3	10^3	10^3
Subclass	IgG2b,κ	IgG2b,κ	IgG1,κ	IgG1,κ	IgG2b,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ
No.	BAS12	BAS13	BAS14	BAS15	BAS16	BAS17	BAS18	BAS19	BAS20	BAS21	BAS22
Titer	10^3	10^3	10^3	10^2	10^3	10^3	10^3	10^2	10^3	10^3	10^3
Subclass	IgG1,κ	IgG2b,κ	IgG2b,κ	IgG2b,κ	IgG1,κ	IgG2b,κ	IgG2b,κ	IgG2b,κ	IgG1,κ	IgG1,κ	IgG1,κ
No.	BAS23	BAS24	BAS25	BAS26	BAS27	BAS28	BAS29	BAS30	BAS31	BAS32	BAS33
Titer	10^3	10^3	10^2	10^2	10^2	10^2	10^2	10^2	10^3	10^3	10^3
Subclass	IgG2b,κ	IgG2b,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG2b,κ	IgG2b,κ	IgG1,κ

No.	BAS34	BAS35	BAS36	BAS37	BAS38	BAS39	BAS40	BAS41	BAS42	BAS43	BAS44
Titer	10^2	10^2	10^0	10^2	10^2	10^1	10^2	10^2	10^3	10^1	10^1
Subclass	IgG1,κ	IgG1,κ	ND	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG1,κ	IgG2b,κ	ND,κ	IgG1,κ
No.	BAS45	BAS46	BAS47	BAS48	BAS49	BAS50	BAS51	BAS52	BAS53	BAS54	BAS55
Titer	10^3	10^4	10^0	10^3	10^2	10^3	10^3	10^2	10^3	10^2	10^0
Subclass	IgG1,κ	IgG2a,κ	ND	IgG1,κ	IgM,κ	IgG1,κ	IgG1,κ	IgG2a,κ	IgG1,κ	IgG2a,κ	IgG2b,κ

Note:ND, undetected.

Table 3. Sequence analysis of ML06 antibody variable domain

McAb chain	V gene %, identity	CDR3 AA length	CDR3 AA sequence
Light chain	IGKV6-32*01, 96.42%	8.3.8	HQDYSSLT
Heavy chain	IGHV2-9*02, 93.68%	8.7.10	ASLPYGRFAY

Table 4. Influence of the interval between toxin and antitoxin injections on survival rate (values in brackets)

Interval	0 h	0.5 h	1 h	2 h	4 h	6 h	Control (no antibody)
Survival rate	5/5 (100%)	5/5 (100%)	5/5 (100%)	3/5 (60%)	1/5 (20%)	0/5 (0%)	0/5 (0%)

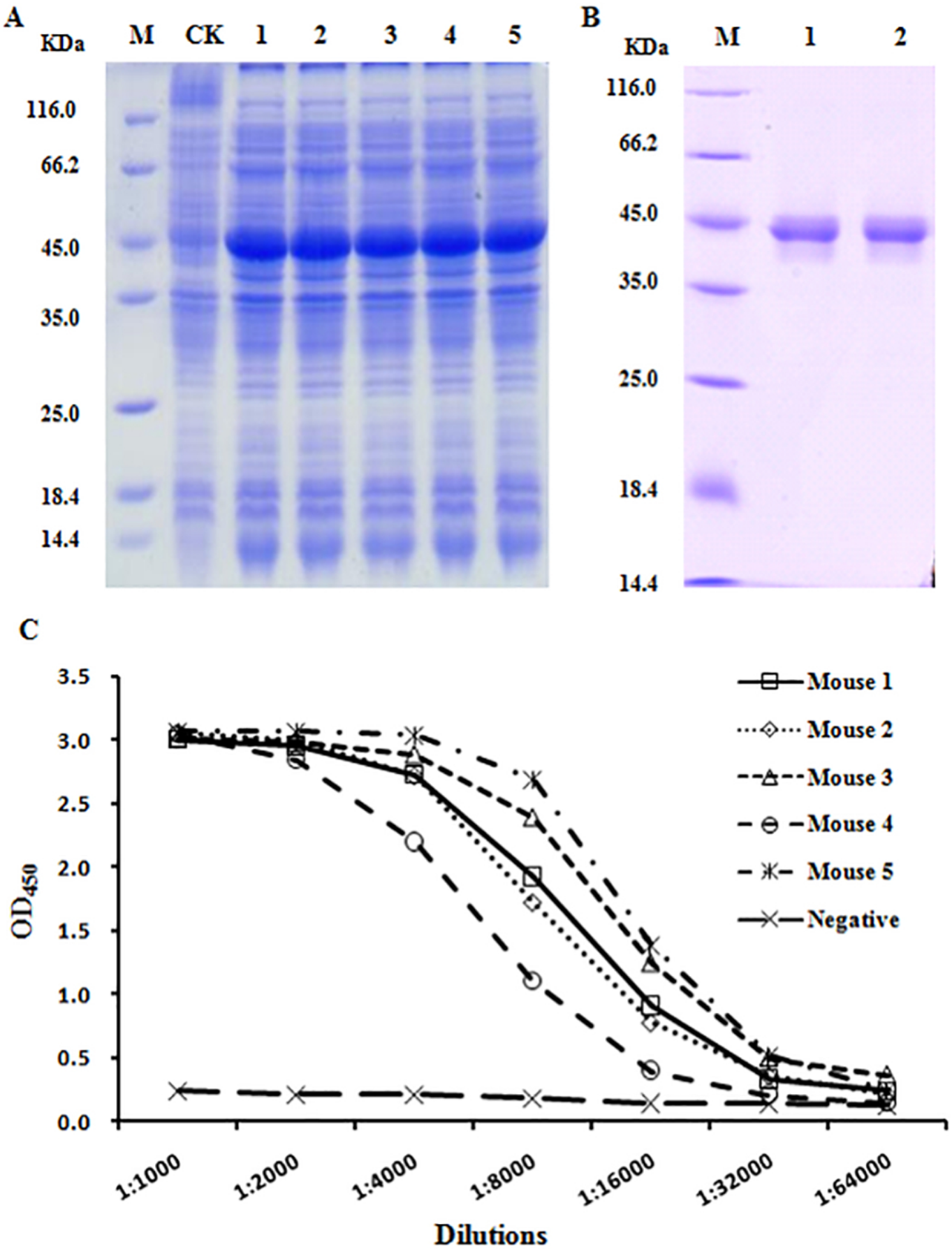
Table 5. Prophylaxis assay of 100 μg/mice ML06 McAb administration (i.p.) followed by injection of 4 LD50 of BoNT/A1 (i.p.) after 0, 1, 3, 7, 14 days.

Interval	0 d	1 d	3 d	7 d	14 d	Control (no antibody)
Survival rate	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	0/5 (0%)

Highlights：

- A novel McAb immunized with recombinant BoNT/AH_C protein was screened and designated as ML06.
- ML06 bound BoNT/A by specifically recognizing a linear epitope of H_{CC} domain of BoNT/AH_C subunit with high affinity.

- ML06 McAb can neutralize the toxin of BoNT/A effectively both *in vitro* and *in vivo*， and would have a good prophylactic capacity for human beings.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4
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14.4
B
KDa
M
1
2
116.0
66.2
45.0
35.0
25.0
18.4
14.4
C
OD450
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3.0
2.5
2.0
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1.0
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Mouse 1
Mouse 2
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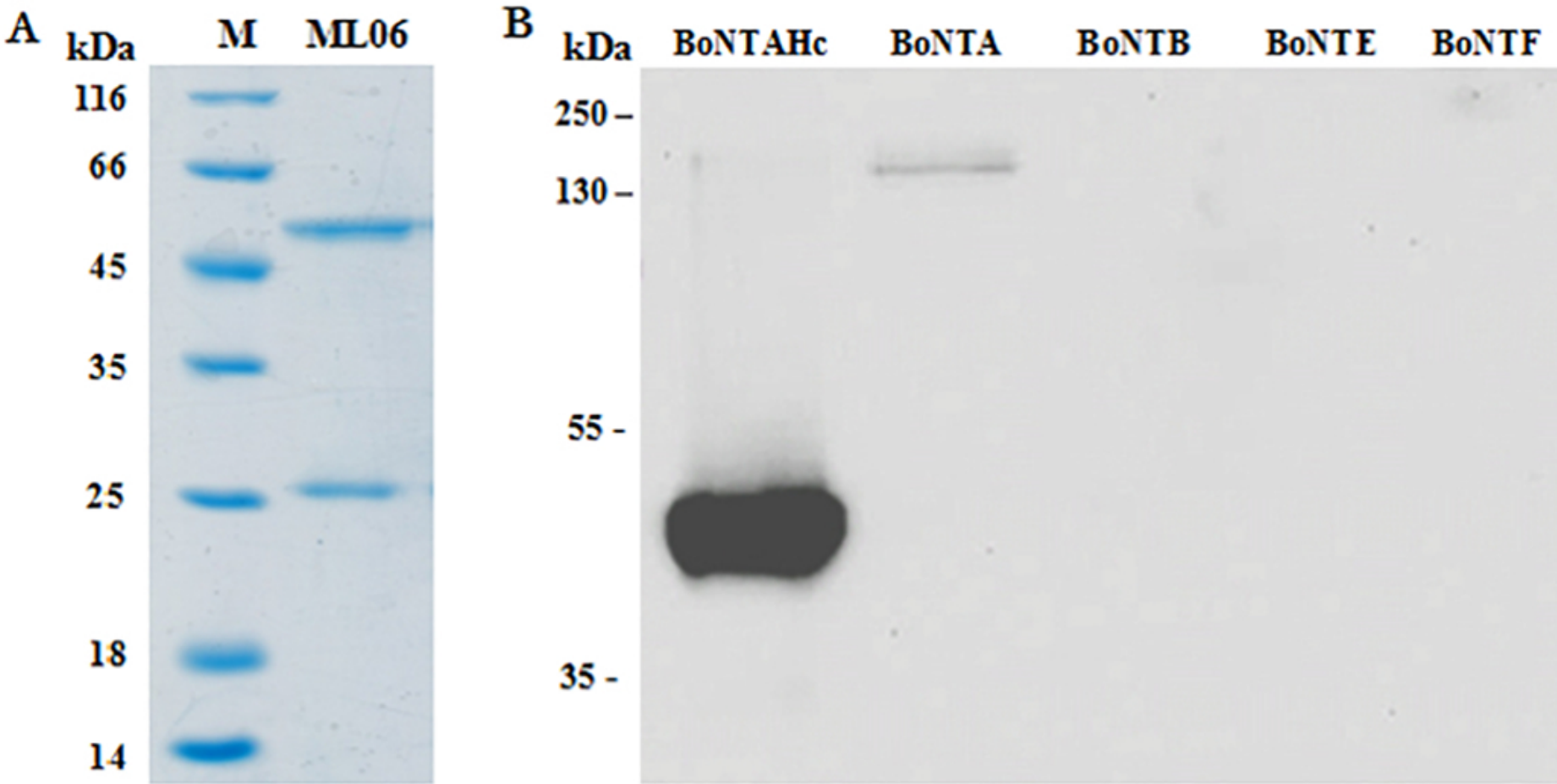
Dilutions

Figure 1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ML06
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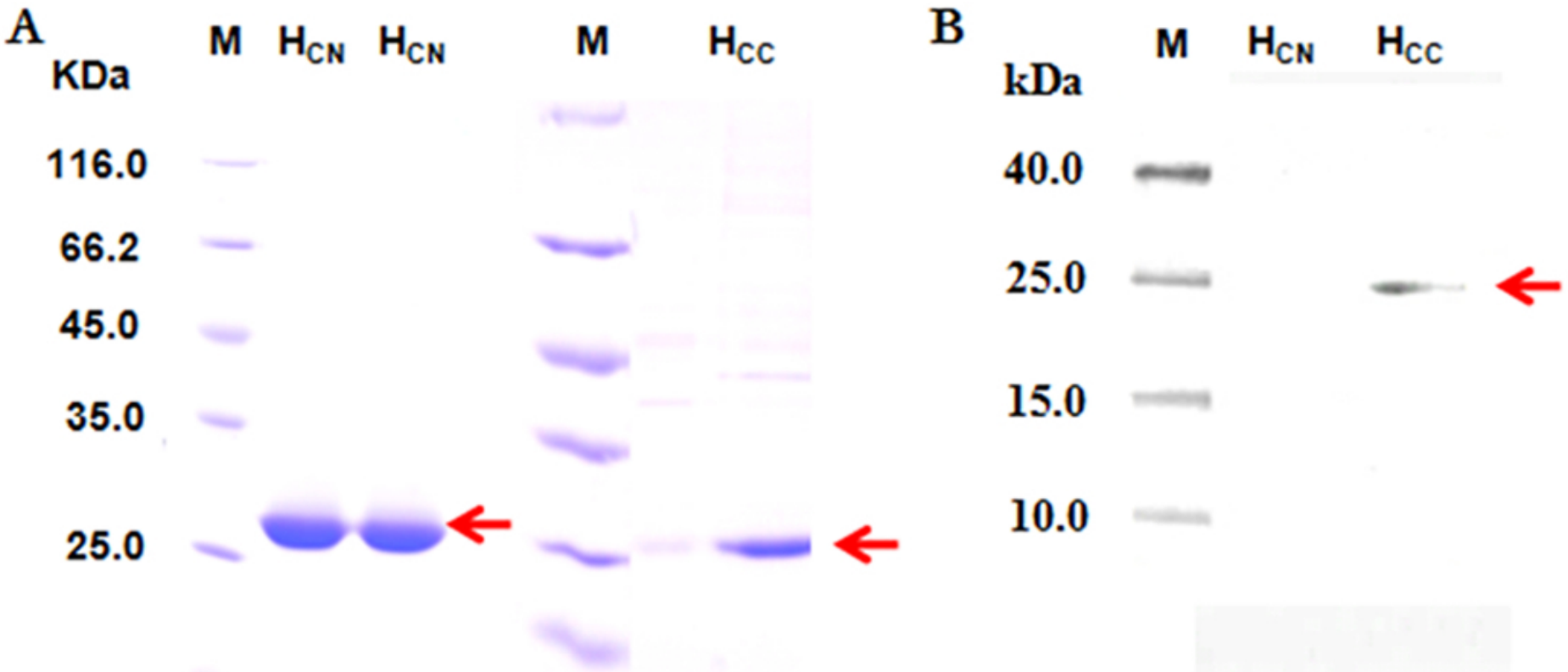
55 -
35 -

Figure 2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10.0

Figure 3

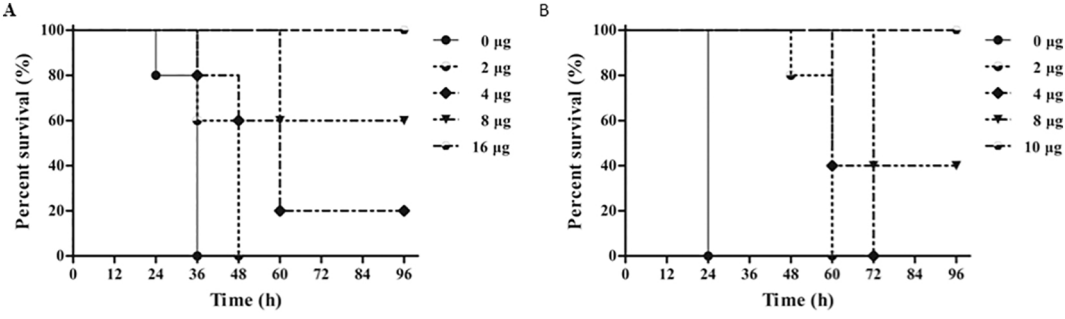
A
Percent survival (%)
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96
Time (h)
0 µg
2 µg
4 µg
8 µg
16 µg
B
Percent survival (%)
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96
Time (h)
0 µg
2 µg
4 µg
8 µg
10 µg

Figure 4