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Cargo-Delivery Platforms for Targeted Delivery of Inhibitor Cargos Against Botulism

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Abstract

Delivering therapeutic cargos to specific cell types *in vivo* poses many technical challenges. There is currently a plethora of drug leads and therapies against numerous diseases, ranging from small molecule compounds to nucleic acids to peptides to proteins with varying binding or enzymatic functions. Many of these candidate therapies have documented potential for mitigating or reversing disease symptoms, if only a means for gaining access to the intracellular target were available. Recent advances in our understanding of the biology of cellular uptake and transport processes and the mode of action of bacterial protein toxins have accelerated the development of toxin-based cargo-delivery vehicle platforms. This review provides an updated survey of the status of available platforms for targeted delivery of therapeutic cargos, outlining various strategies that have been used to deliver different types of cargo into cells. Particular emphasis is placed on the application of toxin-based approaches, examining critical issues that have hampered realization of post-intoxication antitoxins against botulism.

Keywords

Antitoxin; botulinum neurotoxin; neuromuscular junction; paralysis; passive immunization; post-exposure therapeutics; single-chain antibodies; synaptic function; tetanus neurotoxin

1. THE NEED FOR POST-EXPOSURE ANTITOXIN THERAPIES

Extensive genetic, biochemical and structural studies of known intracellularly-acting bacterial protein toxins have provided valuable insights into toxin-mediated disease processes, and this information has enabled scientists to exploit these proteins as powerful research tools in eukaryotic cell biology and as therapeutic agents in biomedical applications [1–8]. Yet, despite the impressive biomedical advances that have been made in our understanding of toxin-mediated disease mechanisms, we still lack a single example of a post-exposure antitoxin therapeutic that can rapidly counteract or reverse any of the known

toxin-mediated diseases once the toxin is already inside the host cell and disease symptoms have manifested.

To date, the only effective preventive measure available against any protein toxin-mediated disease is vaccination to generate toxin-neutralizing antibodies [9–10], and the only prophylactic treatment available after toxin exposure is passive immunization, i.e., neutralization and clearance of circulating toxin by injection of exogenously generated antibodies (usually in the form of horse serum) [11–13]. The problem with this scenario is that once a host cell has been intoxicated, antibodies are no longer effective at neutralizing the toxin or reversing the cytotoxic effects.

The depth and scope of our knowledge of the structure and function of some of the well-characterized toxins has enabled us to gain a better picture of the range of biological functions that can be manipulated by bacterial protein toxins. Indeed, we are now poised to begin contemplating ways to counteract the deleterious effects on the host by a few of these toxins (most notably, anthrax toxin, cholera toxin, Shiga-like toxins, and the clostridial botulinum and tetanus neurotoxins). Efforts are now underway to develop post-exposure antitoxin therapeutics. In this review, we will focus on the current state of development of platforms for targeted delivery of antitoxin inhibitors against botulism and the challenges encountered that have hampered progress.

2. STRUCTURE AND MODE OF ACTION OF CLOSTRIDIAL NEUROTOXINS

Botulinum neurotoxins (BoNTs), serotypes A through H, produced by *Clostridium botulinum*, and the related tetanus toxin (TeNT) produced by *C. tetani*, are one of the groups of toxins, for which tremendous progress has been made in the past couple decades toward our understanding of their structure and function. BoNT serotypes A and B, and to a lesser extent E and F (and now H), are associated with human botulism cases, while serotypes C and D are more prevalent in bird and animal botulism [14]. These toxins are produced as single-chain proteins of approximately 150 kDa and share 3–30% primary amino acid similarity among each other [14–15]. Despite the sequence differences among the serotypes, all these proteins share a common structure and mechanism of action and are considered the most potent toxins for humans.

BoNTs cause flaccid paralysis, while TeNT causes spastic paralysis. Some BoNTs (e.g., BoNT/A) are produced in bacteria as a protein complexed with stabilizing non-toxic neurotoxin-associated proteins [16], but the toxin alone is capable of intoxication [17]. BoNTs are activated by proteolytic cleavage to generate an N-terminal 450 amino acid fragment (light chain = LC, ~50 kDa) comprised of the catalytic zinc-dependent protease domain [18] and a C-terminal 800 amino acid fragment comprised of the translocation and receptor-binding domains (heavy chain = HC, ~100 kDa) [19]. The dichain is held together by a disulfide bond, which is reduced upon translocation into the cytosol of the neuronal cell.

The crystal structures are available for three of the holotoxins, BoNT/A [20], BoNT/B [21], and BoNT/E [22], as well as various domain fragments of these and other serotypes, alone or in complex with their ligands, substrates or receptors [23]. The crystal structure of

BoNT/A (Fig. 1, PDB 3BTA) revealed three functional domains: a receptor-binding domain made up of two sub-domains, a translocation domain consisting of long α -helices and an unusual belt (green in Fig. 1) that wraps around the zinc-containing catalytic domain along the substrate-binding sites and occludes the active site pocket [20, 24].

BoNTs bind to receptors on neurons at the presynaptic ending, undergo receptor-mediated endocytosis, and in the low pH of the endocytic vesicle are translocated into the cytosol of the neuron, where SNARE proteins are subjected to proteolysis by the released zinc-dependent protease domain [25–29]. SNARE stands for soluble N-ethylmaleimide-sensitive factor activating protein receptor. SNARE proteins are a complex of proteins that are involved in membrane fusion of neurotransmitter-containing synaptic vesicles with the plasma membrane at nerve endings and include the proteins SNAP25, VAMP/synaptobrevin, and syntaxin. Proposed models of BoNT intoxication are illustrated in Fig. (2), and involve the following steps: (Step 1) binding of the C-terminal portion (H_C) of BoNT-HC to presynaptic membrane receptors, (Step 2) uptake into an intracellular vesicle, and (Steps 3–6) translocation of the catalytic light chain (BoNT-LC) into the cytosol via the N-terminal portion (H_N) of BoNT-HC. Delivery of the catalytic BoNT-LC into the cytosol then leads to BoNT-LC-mediated proteolytic cleavage of serotype-specific SNARE-containing proteins involved in synaptic vesicle function.

BoNT-LC-mediated cleavage of the SNARE proteins inhibits exocytosis of neurotransmitter-containing synaptic vesicles by preventing fusion of the synaptic vesicles with the plasma membrane and subsequent acetylcholine neurotransmitter release into the synapse. The molecular targets of BoNTs have been defined [27–32]. All BoNTs cleave their substrates at specific peptide bonds: BoNT serotypes B, D, F and G, as well as the related TeNT from *C. tetani*, cleave synaptobrevin (also called VAMP), a membrane protein found in small synaptic vesicles; BoNT serotypes A, C and E cleave SNAP25 at the presynaptic plasma membrane; and BoNT serotype C also cleaves syntaxin at the plasma membrane. The substrate specificity of the newly identified BoNT/H [30–31] has not yet been reported.

Current models propose that the host receptors for BoNTs and TeNT define their ultimate localization in the nerve cell [33]. Binding and endocytosis of clostridial neurotoxins is thought to occur via multiple membrane receptors: gangliosides that bind toxins with low affinity and/or neuron-specific protein co-receptors, the synaptic vesicle membrane protein synaptotagmin (Syt-I, Syt-II) and/or synaptic vesicle glycoproteins (SV2A, SV2B, and SV2C) [34]. These form high-affinity, high-specificity complexes [34], which cluster within arrays of presynaptic receptors at the peripheral nerve terminal [35]. Upon binding, the BoNTs enter cells via receptor-mediated endocytosis and upon endosomal acidification translocate their cargo LCs into the cytosol, where they gain access to their target substrates [26, 36]. A model for LC translocation has been proposed [26], whereby the lowering of pH in the endosome triggers a large conformational change in the translocation domain, resulting in formation of a channel in the vesicle membrane that then mediates translocation of the unfolded LC cargo domain through the channel into the cytosol, where it subsequently refolds. Whether and at what point further processing and dissociation of the receptor-

binding domain (BD) from membrane-translocation domain (TD) occurs during the translocation process is not clear.

3. NEUROMUSCULAR PARALYSIS BY CLOSTRIDIAL NEUROTOXINS

The intravenous lethal dose of BoNT/A is estimated to be 1–5 ng/kg [32, 37–38]. Symptoms of botulism paralysis are due to the inhibition of acetylcholine neurotransmitter release from peripheral motor nerve endings, which results in flaccid paralysis [28, 38–39]. Unless respiration is facilitated by mechanical ventilation, death occurs from respiratory failure due to neuromuscular paralysis [37, 40]. The duration of paralysis depends mainly on the serotype of the BoNT [39, 41]. When injected into humans, BoNT/A, B, C1, E and F cause neuromuscular paralysis for distinct periods of 4–6, 3, 4–5, 1 and 2 months, respectively [42–45]. Importantly, although the release of neurotransmitter is impaired, the original neuromuscular synaptic connection to the muscle remains intact [42–46]. This lends hope for possible recovery of neuromuscular function after BoNT intoxication in the event an inhibitor that blocks toxin action could be delivered inside the motor neuron.

4. TOXIN-BASED THERAPEUTICS

4.1. Biomedical Applications of BoNTs

Inhibition of neurotransmitter release is not cytotoxic to neuronal cells; rather, the toxicity of BoNT is due primarily to the vital nature of neuronal transmission for the overall function of the whole organism. With the exception of BoNT/C, which is cytotoxic to cultured neuronal cells [47–48], BoNTs can be used as therapeutic agents to treat various neurological disorders of dystonia and spinal spasticity [49–50]. Indeed, BoNT/A (Botox™) and BoNT/B (Myobloc™) are both approved by the FDA for cosmetic applications or for therapeutic use to treat neuromuscular and secretory disorders.

A concern inherent of any protein-based therapeutic is the potential for the host to elicit an immune response against the foreign protein [51], leading to decreased effectiveness of the therapeutic, causing immunoreactivity such as hypersensitivity and anaphylaxis, and/or resulting in altered pharmacokinetics of the therapeutic [52]. Typically, low doses of BoNT/A used in therapeutic or cosmetic applications do not elicit any significant neutralizing host response, but in cases of prolonged usage BoNT/A can become ineffective [53]. Progress has been made toward developing technologies to humanize antibodies and other protein-drug conjugates for use as therapeutic reagents [51, 54–55], such as removal of problematic B- and/or T-cell epitopes [6, 56]. These technologies have addressed many of the concerns associated with use of proteins as biological drugs and could be employed toward improving BoNT-based applications.

4.2. Immunotoxins

The development of immunotoxins has a history of over 30 years [57]. Much of the lessons learned from these studies can be applied to the development of BoNT-directed therapies. Immunotoxins are intended to serve as cytotoxic killing agents that can be specifically delivered to cancer cells with the aide of cell-targeting proteins such as antibodies. The cytotoxic potency of the catalytic domains of diphtheria toxin (DT) and ricin were the first

to be employed as warheads for targeted killing of cancer cells, using a tumor cell-targeting antibody chemically conjugated to the catalytic domain of the toxin [58]. Early immunotoxins prepared through chemical coupling techniques displayed several undesirable characteristics, including batch-to-batch heterogeneity, poor stability, and low yields. With the introduction of molecular cloning techniques, the killing domain could be directly coupled with the tumor-cell targeting domain via peptide linker as a recombinant fusion protein that was engineered optimally for expression and stability in *E. coli*. Unlike the early immunotoxins produced through chemical conjugation, the recombinant fusion proteins could be obtained with uniform molecular integrity, high purity, and in large quantities.

For example, an engineered immunotoxin consisting of the active fragment of *Pseudomonas* exotoxin A (PE40) fused to two linked antibody variable domains (VHVL), derived from a monoclonal antibody directed against the human interleukin-2 (IL-2) cytokine receptor, was first produced and purified as a recombinant protein (IL-2-PE40) in *E. coli* [59]. Similarly, a toxin catalytic domain, such as the A fragment of DT (DTA), could be fused with a tumor cell-targeting polypeptide, such as the cytokine IL-2, to generate a recombinant immunotoxin DTA-IL-2, which could be expressed and purified from *E. coli* [60]. This enabled specific targeting of the cell-killing moiety (PE40 or DTA) to a tumor cell via cell surface cytokine receptors that would be upregulated in the tumor cell. Other recent efforts have involved utilization of the binary anthrax lethal toxin from *Bacillus anthracis* to deliver cytotoxic enzymes, such as PE40, to the cytosol of tumor cells [61]. Several of the clostridial binary actin-ADP-ribosylating toxins have a delivery system similar to anthrax toxins and have been explored as cargo-fusion proteins for transporting proteins into the cytosol [62].

The more recent advances in antibody research ushered in the technology for generating single-chain antibodies (scFv) and single-domain antibodies, such as those derived from camelid antibodies, VHHs or nanobodies [63]. These relatively small (~14-kDa), soluble and stable antibodies have revolutionized the area of recombinant immunotoxins. Coupling DT, PE or ricin activity domains to these single-domain binding moieties enables more biomarkers to be used for highly selective targeting of many different types of cancer cells [6, 64–67]. Many of the strategies used in developing current immunotoxin therapies are intended for killing cancer cells, and the therapeutic objective can be achieved so long as the toxin catalytic domain can reach its cellular target, i.e., the protein synthesis machinery. An ideal post-intoxication anti-botulism therapy, on the other hand, would need to be highly specific not only for its target cells, but also for blocking the action of the intracellular BoNT-LC molecules without causing any adverse off-site effects. In terms of adverse reactivity, there is substantial, accumulating clinical evidence from BoTox formulation and evaluation studies that indicate BoNT-derived therapies are well tolerated and have low immunogenicity rates [68–71]. BoNT-based delivery platforms might thus be well suited for therapeutic applications, as they may not elicit robust immune responses.

4.3. BoNT-LC-Chimeras for Therapeutics

Just like for immunotoxins, the Zn^{2+} -dependent protease activity domain of BoNTs could be delivered through a heterologous receptor-targeting cargo-delivery domain to cells that do

not have receptors for the BoNTs. In this fashion the range of BoNT therapeutic potential can be extended to non-neuronal cells as well, in particular secretory cells and sensory neurons [72–73]. Additionally, engineered chimeric BoNT toxins, where domains displaying selective properties are swapped among the BoNT serotypes, are being developed as anti-nociceptive therapeutics to treat chronic pain and other secretory disorders [50]. For example, BoNT/E-LC strongly inhibits the release of calcitonin-gene-related peptide (CGRP) from sensory neurons and suppresses subsequent excitatory effects that are associated with chronic pain, but there are many more receptors for BoNT/A-HC on sensory neurons than for the targeting domain of BoNT/E-HC. By coupling the activity of BoNT/E-LC with the sensory neuron-targeting domain of BoNT/A-HC, the resulting chimeric toxin was effective in alleviating chronic pain [74].

5. CURRENT ANTITOXINS AGAINST BOTULISM

5.1. Distinction Between Antitoxins that Block Toxin Uptake and Antitoxins that Mediate Post-Intoxication Recovery

Current anti-botulism strategies are prevention through vaccination [75] or neutralization of circulating toxin through passive immunization [37, 76]. Passive immunization usually involves administration at early stages of intoxication with neutralizing antibodies derived from horse anti-sera [11] or in the case of infant botulism from human-derived immunoglobulins [77]. The serious problem of anaphylaxis in intoxicated individuals has now been ameliorated by the development of despeciated antibodies, where the Fc region is removed from immunoglobulins derived from horses immunized with toxoid or toxin. The only antitoxin currently used in the U.S. for naturally occurring non-infant, food-borne botulism is a heptavalent antitoxin against BoNT/A-G (HBAT), comprised primarily of Fab and F(ab')₂ immunoglobulin fragments, which is available from the CDC. To reduce risk of anaphylaxis in cases of infant botulism, a human antitoxin is available, called “Baby-BIG”, which is derived from human donors who received the pen-tavalent (BoNT/A, B, C, D and E) vaccine [77–78]. Alternative antitoxins based on camelid VHH antibodies, which provide good production, superior heat stability, and solubility, have also been explored recently [79–81].

One of the greatest challenges in developing post-exposure treatments for botulism is the short therapeutic window that is available for administration of antisera [37, 43]. Immunotherapy must be administered promptly (within 24 hours) after onset of symptoms, but before respiratory distress occurs, when toxin is still accessible to the antibody. In addition, it should be noted that because of the high diversity among the BoNT serotypes, antibodies generated against one serotype are not protective against the other serotypes. Indeed, the recent clinical isolation of a new serotype (BoNT/H) [30–31], which may be as potent as BoNT/A, yet is not neutralized by antisera from any of the other serotypes, illustrates the challenge of developing universal antitoxins based on neutralizing antibodies against all of the serotypes.

Neutralizing antitoxin prevents the toxin from binding to target neuronal cells and allows for rapidly clearance of toxin from the bloodstream. However, antitoxin is effective only when given before onset of paralysis. Unfortunately, intoxicated individuals are unlikely to be

aware that they have been exposed to toxin until they become symptomatic. Administration of antitoxin does not reverse the course of disease; it only halts the progression of severity. To date, there is no effective antidote available for recovering from paralysis after symptoms have occurred [37, 76].

Even with antitoxin administration, recovery is slow [37, 43], with the length of time required to recover neuromuscular function dependent on the type of nerve terminal affected, the serotype of BoNT involved, and the dose of toxin [82]. Indeed, recovery after exposure to BoNT/A typically requires 2–6 months depending on dose, but can take much longer (>1 year). During this time patients are still paralyzed and require respiratory and other mechanical support [76, 83].

5.2. Use of Toxin-Based Vehicles for Targeted Delivery of Inhibitory Cargos

Since the neurons are not killed from botulism and muscular atrophy is the main concern with prolonged paralysis [76, 83], the primary goal for post-exposure treatment of botulism is to shorten the recovery time from the typical 2–6 months to perhaps just a few days. For this post-intoxication therapeutic strategy to succeed, it is critical that the therapeutic agent is targeted to the correct cells, since it would not only be inefficient, but also could cause adverse side effects if delivered to the wrong cells.

One solution to this problem is to deliver toxin-neutralizing inhibitors or antibodies to the site of intoxication at the nerve terminal by using the BoNT-HC, which specifically targets neuronal cells. Indeed, BoNT-HCs are already designed to transport large polypeptide cargos (e.g., BoNT-LCs with molecular masses of ~50 kDa) across cellular membranes. The advantage of using the native BoNT entry pathway for delivery is that the cargo will be transported to the precise site of the target. Importantly, this antitoxin strategy has the potential to reverse and/or shorten the duration of the clinical effects of intoxication (i.e., paralysis). Proof of this concept was demonstrated through using neutralizing antibodies, delivered via electroporation, to block BoNT/A- or TeNT-dependent inhibition of exocytosis in chromaffin cells [84]. In light of this, it is conceivable that replacing the LC with inhibitory cargos, tethered to the BoNT-HC, containing both TD and BD, could block toxin action and promote recovery from paralysis.

5.3. Inhibitory Cargos

In terms of potential inhibitory cargos that might be delivered into a neuronal cell, there are several aspects that must be considered. Any candidate inhibitory cargo must be of sufficient selectivity to target only the action of the toxin, not other host zinc-dependent proteases, and must be of sufficient potency to compete with the native toxin activity or to eliminate the toxin so that it can no longer act on new substrate. Several possible strategies have been developed that involve either inhibition of the BoNT-LC or replenishing the respective substrate (SNAP25, VAMP, or syntaxin).

A number of laboratories have focused on identifying small molecule inhibitors that block BoNT catalytic activity with high specificity and high affinity [85–89]. These approaches rely on good high-throughput screening approaches to identify lead compounds that can then

be further screened using additional *in vitro* and cell-based assays, followed by *in vivo* animal studies. Unfortunately, to date only a few small molecule compounds have been identified as lead candidates with good *in vitro* potencies that are also active in cell-based and *in vivo* assays [85–86, 90–91]. Most of these reported small molecule inhibitors of BoNT/A-LC are hydroxamate-based zinc-chelators, with the most potent hydroxamate compounds having K_i values as low as 27 nM, but these candidate inhibitors generally showed poor bioavailability, short *in vivo* half-lives, and unacceptably high cytotoxicity [86, 89–90]. Modification of these compounds for improved *in vivo* properties results in decreased potency with the most potent compounds having an IC_{50} value of 230 nM [91] or a K_i value of 400 nM [87]. Alternative quinolinol-based compounds have been reported with good ex vivo properties in cell-based and tissue-based assays, with IC_{50} values of 800 nM, albeit with poor solubility at neutral pH [85].

Somewhat larger cargos that block toxin catalytic activity include peptide-based pseudosubstrates or peptidomimetic inhibitors have been designed to ameliorate neuronal selectivity and substrate specificity. Several high-affinity competitive inhibitors have been identified that are based on peptides that bind to the active site. Residues flanking the cleavage site of SNAP25 have been used to design small peptide-based inhibitors, including CRATKML-NH₂ [92–93], RRGL-NH₂ [94], and related peptides. N-acetyl-CRATKML-NH₂ and its derivatives are effective against BoNT/A with K_i values as low as 330 nM [95]. A number of substrate-based tetrapeptide inhibitors against BoNT/A have been explored, with an RRGC tetrapeptide showing the best inhibition of BoNT/A-mediated SNAP25 cleavage in mouse brain lysates [94, 96], with the most potent tetrapeptide inhibitor RRGF exhibiting an IC_{50} value of 900 nM and a K_i value of 358 nM [97]. VAMP substrate-based peptides, such as VAMP-2(32–65) with D-Cys in place of Gln-58, are effective against BoNT/F with K_i values as low as 1 nM [98–99]. Of a series of non-zinc-chelating, non-hydroxamate-based, peptidomimetic inhibitors designed with structures resembling the topology of the cleavage site (residues QRATKML) of SNAP25 when bound to the BoNT/A active site, one inhibitor denoted as I1 exhibited a K_i value of 41 nM [100].

Several pseudo substrate Q197-R198 cleavage-site mutants for SNAP25 (i.e., R198A, R198E, R198T and A195S) have been identified that are resistant to BoNT/A-LC-mediated proteolysis [101–103]. A series of alternative 66-mer, pseudosubstrate inhibitor peptides, denoted as SNAPi, have also been reported that capitalize on the extended BoNT/A substrate-recognition sequence conferred by both exosites within the substrate sequence (residues 141–206) of SNAP25 [104]. In this series of SNAPi, up to five Gly residues were inserted at the Q197-R198 cleavage site to pack the active site cleft. SNAPi with 1 Gly or 3 Gly insertions were 10-fold more effective inhibitors than wildtype 66-mer peptide, with IC_{50} values of 1.3 and 1.7 μ M, respectively, and importantly both were highly resistant to toxin cleavage.

Another type of inhibitory cargo might target the catalytic activity of the BoNT-LC by blocking the active site with a tight-binding, activity-neutralizing antibody in the form of engineered scFvs or camelid VHH antibodies. Several VHH antibodies against BoNT/A-LC and BoNT/B-LC have been cloned from immunized alpacas that bind LC with high affinity [81, 105], some of which also blocked toxic activity. There are other BoNT/A-LC-

neutralizing VHH antibodies derived from llamas available, one of which has been crystallized as a complex with BoNT/A-LC and shown to bind to the α -exosite of the toxin [106]. Additional BoNT/A-LC-specific inhibitory VHH antibodies have been cloned from camels [107]. A high-affinity, BoNT/A-LC-neutralizing human monoclonal antibody is also available [108], which could be converted into an scFv or Fab/F(ab')₂ antibodies.

Alternatively, the cargo might be a rescue package designed to restore functionality by providing a noncleavable but fully functional version of the SNARE proteins. For this approach, the inhibitory cargo would be introduced via exogenous expression of the protein in the neuronal cells using a mammalian expression vector. Feasibility for rescue of neurotransmitter release by delivery of a cleavage-resistant SNAP25 mutant was first demonstrated in BoNT/A-intoxicated cultured chromaffin cells transfected with a plasmid encoding the cleavage-resistant SNAP25 mutant [102]. This approach could also be achieved through delivery of a viral expression vector, such as a replication-deficient adeno-associated virus (AAV) containing a gene encoding a noncleavable SNAP25 mutant [103] or a lentivirus containing the genes encoding the noncleavable SNAP25 mutants [109]. In this latter study, a lentivirus, encoding a gene for SNAP25 with three mutations (D179K, M182T, and R198T) that render the SNAP25 protein noncleavable by BoNT/A, BoNT/C or BoNT/E protected neuronal cells from BoNT/A and BoNT/E, although it appeared to counteract against BoNT/E inhibition more effectively.

Another strategy is to stimulate clearance of the BoNT-LC or BoNT-LC-inhibitor complex within intoxicated neuronal cells by targeting the LC for degradation through the E3-ligase-proteasome pathway [41]. In this approach, a functional module that would lead to more rapid degradation of the intracellular LC would be incorporated into the LC-targeting moiety of the delivered cargo. For example, transfection of an E3-ligase domain (HECT domain from E6AP or RING domain from XIAP) coupled with a cleavage-resistant version of SNAP25 (R198T) into neuronal cells promoted the polyubiquitination of BoNT/A-LC, which shortened the intracellular half-life of the LC [110]. Accelerated degradation of BoNT/A-LC and BoNT/B-LC by the ubiquitin proteasome system was also achieved through transfection with fusion proteins consisting of the LC-binding camelid antibodies VHH-B8 and VHH-B10, respectively, linked to the F-box domain region of (β -TrCP, which forms a multimeric E3 ubiquitin-ligase complex with Skp1 and Cullin [111].

5.4. The C Terminus of TeNT as a Delivery Vehicle to the Central Nervous System

TeNT shares structure similarity with BoNTs and likewise binds to the presynaptic membrane. The major difference is that unlike all BoNTs, which translocate their LC cargos immediately after cellular uptake and thus act primarily at motor nerve termini, TeNT travels further along the axon and transsynaptically migrates to the central nervous system (CNS) [33]. The C-terminal binding domain of TeNT (denoted as TTC) has been explored as a delivery vehicle to the CNS [112]. A beta-glycosidase(β -gal)-TTC hybrid was first obtained as a recombinant GST-affinity-tagged fusion protein from *E. coli*. After intramuscular injection of this (β -gal-TTC protein in mice, uptake and retrograde migration to the CNS was visualized by histological X-Gal-staining [113]. Similarly, uptake and the migration of a recombinant GFP-TTC fusion protein in the CNS were demonstrated in the

brain after injection of Neuro2A neuroblastoma cells ectopically expressing GFP-TTC or adenovirus encoding GFP-TTC into the striatum [114].

Although TTC has been conceptualized for drug entry into the CNS, both β -gal TTC and GFP-TTC are localized in vesicle compartments in the target neuronal cells and may not be accessible to other subcellular locations. Biochemical analysis of detergent-extracted neuromuscular preparations showed that the GFP-TTC fusion protein was associated with detergent resistant membrane fractions and not the cytosol [115]. Several fusion proteins have been tested and reported to have some effects on neuronal cells, including Bcl-xL-TTC [116], BDNF-TTC [117], and GDNF-TTC [118]. However, the TTC delivery platform itself without a fusion cargo also stimulated signaling pathways [117, 119], which complicated the interpretation of the results.

6. RATIONAL APPROACHES TO ANTI-BONT CARGO-DELIVERY PLATFORMS

A possible strategy to address the problem of solubility, bioavailability, and neuron-targeting properties of candidate small molecule, peptide-, or antibody-based inhibitors might be to conjugate or fuse them with a neuronal-specific cargo-delivery vehicle. Alternatively, these inhibitors could be encapsulated in liposomes for delivery [111, 120–121], provided they have a neuronal-targeting delivery vehicle also incorporated into the design. Considering our current understanding of the BoNT intoxication mechanism (Fig. 2) and information gleaned from the TTC-related studies described above, strategies for rational design of anti-botulism drug-delivery platforms can be sorted into at least four scenarios: (a) cell-penetrating peptide-based approaches, (b) piggybacked holotoxin-based approaches, (c) toxin binding-domain-based endosomal delivery approaches, and (d) retrograde transport-based approaches (Fig. 3). It is also conceivable that a combination of multiple strategies from the above could be employed to achieve a practical solution for an ideal cargo-delivery platform.

6.1. Cell-Penetrating Peptide-Based Approaches

The development of cell-penetrating peptides (CPP), or protein transduction domains (PTD), for delivery of therapeutic cargos has a history of more than 25 years, since the initial discovery of the transactivator of transcription (TAT) protein of the HIV virus [122]. The CPP-based delivery strategy has been demonstrated successfully for delivery of various forms of biomolecules, including small molecules, oligonucleotides, siRNA, plasmid DNA, peptides, proteins, liposomes and nanoparticles. There have been many reviews written on the applications of CPP-based delivery platforms (see reviews [123–125]). Considering that a direct fusion of a CPP with an inhibitory scFv antibody [126] or a VHH protein [127] has already been reported as an antiviral delivery platform, a similar CPP-scFv or CPP-VHH for neutralizing each serotype of BoNTs should likewise be feasible. However, such CPP-based delivery platforms for anti-botulism cargos would need to incorporate a mechanism for achieving specificity to target the intended neuronal cells.

6.2. Piggybacked Holotoxin-Based Approaches

Taking advantage of the route for BoNT entry pathway, it would seem to be an ideal approach to simply piggyback the therapeutic cargo directly onto the LC cargo at the N terminus. When the LC is translocated from the endosome into the cytosol the piggybacked cargo will be translocated together with the LC, accessing the same location that the toxin does. This could be appealing in light of the fact that several enzymatically inactive mutants of the LCs have been identified.

This piggybacked approach was first reported using full-length BoNT/D as a delivery vehicle, where the protein cargo, GFP, dihydrofolate reductase, luciferase or BoNT/A-LC, was fused to the N terminus of the holotoxin [128]. In this study, delivery of BoNT/A-LC into the cytosol of isolated synaptosomes was detected through cleavage of SNAP25, while presence of other piggybacked cargos in the cytosol was implicated through BoNT/D-LC-mediated cleavage of synaptobrevin (VAMP). Methotrexate-complexed dihydrofolate reductase as a piggybacked cargo reduced the activity of BoNT/D-LC [128], presumably due to inhibition of cargo unfolding during the translocation process. In line with the model where unfolding/melting of the cargo domain takes place during translocation [129], the GFP piggybacked cargo also reduced BoNT/D-LC activity. In the absence of evidence showing the integrity of the fusion protein in endosomes and the delivered cargo after translocation, there could have been other interpretations for the observed delivery of enzyme activity into synaptosomes. A fusion of BoNT/E-LC with full-length BoNT/A was also shown to efficiently deliver both the BoNT/E-LC cargo along with the BoNT/A-LC with full activity [130].

A key aspect of a piggybacked strategy for delivery of therapeutic cargos is the requirement for a completely inactive mutant form of the holotoxin as the delivery vehicle. It has been shown that a single mutation (E244Q) in the active site of BoNT/A still cleaves SNAP25, whereas a double mutant (E224Q/Y366F) is catalytically inactive and forms a stable complex with SNAP25(141–204) peptide substrate [131]. Additional enzymatically inactive BoNTs have been reported, including BoNT/A^{RYM} (R363A/Y365F) [132], ci-BoNT/A1 (H223A/E224A/H227A) [133], BoTIM/B (E231A/H234Y) [109], LH_N/A (E224Q and H227Y) [73], and TeTIM (E234A) [134]. All these inactive mutants could have dual use for both developing vaccines as well as cargo-delivery platforms.

For the purpose of selectively activating the engineered holotoxin proteins into dichain products to facilitate delivery, selective cleavage sites have been incorporated into the recombinant toxin proteins, including substrate sequences of factor Xa or enterokinase [135], TEV protease [136], and even the SNARE substrate sequences for BoNTs [73]. It was demonstrated that using the piggybacked mutant holotoxin approach, a core streptavidin (CS) moiety was delivered into cultured spinal cord neurons by coupling catalytically inactive CS-BoTIM/B holotoxin fusion protein with biotinylated lentiviral vectors [109] or biotinylated liposomes [137]. A similar neurotropic delivery vehicle intended for retro-axonal transduction of viral vectors based on the catalytically inactive CS-TeTIM holotoxin fusion protein has also been reported [134].

6.3. Toxin Binding-Domain-Based Endosomal Delivery Approaches

Full-length BoNTs and TeNT have been reported to deliver several conjugated cargo proteins into neurons, as discussed above, and holotoxin-based platforms may have advantages in the case of TeNT-derived delivery vehicle for trans-axonal delivery, as suggested by recent work [134, 138]. However, attaching heterologous inhibitory cargo to a large-sized, full-length holotoxin (150 kDa) poses some difficulty in preparing stable and soluble fusion proteins. Challenges associated with protein expression, as well as safety and biosecurity considerations (i.e., the requirement for costly CDC-approved biosafety level 3 containment facilities and protocols for production in the U.S.), hinder the production and development of the full-length recombinant proteins for therapeutic applications, particularly those requiring large quantities of the protein [75].

For most BoNTs, the BD is the primary determinant for neuronal internalization and the TD mediates delivery of the cargo into the cytosol. It is thus easy to envision that replacing the zinc-dependent protease LC with an inhibitory antitoxin cargo would simplify the protein production and toxicity issues associated with piggybacked approaches, while still using the TD-BD function to deliver the cargo to the precise location where the internalized toxin resides. Challenges that would have to be overcome with such an approach would be designing an appropriate linker moiety that could be used to fuse the delivery vehicle with the inhibitory cargo, incorporating a mechanism for release of the inhibitory cargo once it is translocated inside the neuronal cell, and including a neuron-targeting and binding component into the delivery vehicle.

The first demonstration of BoNT-HC-dependent nontoxic cargo uptake into neuronal cells was achieved using a chemically modified native BoNT/A-HC [139]. Construction of this early BoNT/A-HC delivery vehicle system utilized a bifunctional chemical crosslinker to conjugate a 10-kDa amino dextran to an HC protein prepared from the native dichain holotoxin. The cargo and delivery vehicle were labeled separately with different fluorescent dyes to allow direct detection of uptake and colocalization of cargo and HC in the cultured neuronal cells [139]. This dextran-HC conjugate has potential for carrying multiple small molecule drugs; however, little evidence supports the feasibility of translocating a drug-decorated dextran molecule into the cytosol.

Recombinant proteins building on the BoNT/A-HC as a delivery vehicle have been challenging, mostly due to inclusion body formation and proteolytic cleavage in *E. coli*. Until recently, whenever production of recombinant proteins with potential cargo fused with truncated versions of the BoNTs or TeNT has been attempted, only fusion proteins with the BD alone could be stably expressed in *E. coli* [112–113]. These cargo+BD constructs lack the TD critical for facilitating delivery of cargo into the cytosol. This difficulty has led a number of researchers to resort to alternative, but less convenient and low-yielding expression systems, such as the Sf9 baculovirus system [136]. The first reported GFP-HC cargo-delivery vehicle, LC-GFP-BoNT/A^{tev} was produced in a baculovirus/sf9 cell system, and used poly-His-tag and strep-tag for purification [136]. More recently, fusion proteins using a synthetic, codon-optimized gene of the BoNT/A-HC fused to GFP as a

prototype cargo with various inter-domain linkers were engineered for *E. coli* expression and purification of soluble and stable recombinant proteins in good yield [140].

In the LC-GFP-BoNT/A^{tev} construct, the GFP gene was connected to Asn391, retaining the belt and inter-chain disulfide bridge loop region, and the cleavage site between the LC and HC was replaced by a tobacco etch virus (TEV) protease cleavage sequence for controlled cleavage [136]. Retaining the belt and inter-chain disulfide bridge loop region in this construct might facilitate delivery and release of cargo, which has been proposed to be important for translocation [129]. Unlike for the native holotoxin, where the belt is stabilized by the presence of LC, inclusion of the belt (residues 390–544) in a fusion protein lacking the LC undoubtedly contributes to instability of the recombinant protein during expression.

An alternative approach would be to bypass the belt and the inter-chain disulfide bridge and connect GFP directly to the start of the TD (at Leu544) or to the start of the BD (at Thr875) with or without an inter-domain linker. Versions of such GFP-containing fusion proteins (GFP-TD-BD and its corresponding GFP-BD) were expressed as soluble proteins in *E. coli* [140]. This approach has the advantage of scale-up, since it only requires His₆-tagged affinity and ion-exchange chromatography. Moreover, both GFP-TD-BD and GFP-BD demonstrated targeted uptake by presynaptic motor neurons at neuromuscular junctions *ex vivo* and *in vivo* [140]. Although there was no known cleavage-release mechanism in the GFP-TD-BD or GFP-BD fusion protein, delivery of GFP into the cytosolic fraction from either GFP-TD-BD or GFP-BD was suggested from western blots for GFP (Ho and Wilson, unpublished results). GFP itself has been reported to have membrane-penetrating function that is tunable through manipulation of surface charge [141]. However, a release mechanism would still be required when other protein cargos are to be delivered by a vehicle through direct fusion at Leu544 or Thr875.

A further reduction in the design of a BoNT-based delivery platform would be to retain only the BD of BoNT and employ alternative translocation machinery for cargo delivery. Such an attempt was reported for a fusion construct based on the *Clostridium difficile* toxin B (TcdB) [142]. TcdB is a 270-kDa protein that consists of a glucosyltransferase (GT) cargo domain, an autocatalytic cysteine protease domain (CPD), a TD and a BD. Here, the BD was replaced with the BD of BoNT/A (residues 861–1296) and an alkyltransferase (AGT) cargo was appended to the N-terminal GT with the intent that the autocatalytic CPD could carry out cargo release into the cytosol. The resulting 281-kDa fusion protein assembly, AGT-TcdB(GT-CPD-TD)-BoNT/A(BD), was tested on neuronal cells and Vero cells for selectivity. Results demonstrated the feasibility of engineering cargo-delivery vehicles with altered target cell-binding specificities and cargo-delivery mechanism, but the approach was marred by the failure to obtain good quality and yields of the large fusion protein [142].

6.4. Retrograde Transport-Based Approaches

A number of bacterial toxins, including Shiga toxin (Stx), cholera toxin (CT), *Pseudomonas* exotoxin A (PE), and the plant toxin ricin, enter their target cells through receptor-mediated endocytosis and translocate their enzyme cargoes after retrograde transport to the Golgi or ER [4, 143–145]. This retrograde delivery pathway is an alternative to the acidification-

dependent endosomal delivery pathway. The efficiency of this retrograde delivery can be further modulated through ER-retention using a C-terminal KDEL sequence [146–147].

In this approach, using CT as an example, cargo delivery could be achieved by fusing it to the catalytic A domain that normally docks onto the pentameric B domain complex. This would then allow for release of cargo after translocation from the ER to the cytosol, presumably through the sec61 translocon. An application of this strategy has been reported [148], where the cell-killing catalytic domain of DT was coupled to CT-A, thereby converting the modified CT into a cytolethal toxin. Alternatively, again using CT as an example, the cargo could be transported through a direct fusion to the B domain. This approach has been used primarily for cancer therapy [149–150] and vaccine antigen delivery [151–152]. For a retrograde transport-based delivery that is neuron specific to be realized, a neuron-specific binding domain must be incorporated into the delivery vehicle. It is also not clear if the retrograde pathway will have selectivity for delivery at the desired motor nerve ending over transaxonal retrograde transport.

CONCLUDING REMARKS

An optimal antitoxin delivery vehicle must be directed against a specific marker on the target cells, in the case of neuronal cells, neuron-specific gangliosides and/or synaptic membrane proteins, such as those that serve as co-receptors for the binding domains of BoNTs. Much of the details regarding binding to the cognate receptor(s) has been elucidated for each BoNT serotype [26, 34, 153–155]. This repertoire of information now provides a good entry point for toxin BD-based delivery vehicles. Alternative neuron-specific VHH-based delivery vehicles analogous to immuno-toxins [57, 65, 156–158] and antibody-drug conjugates [159–160] could also be adapted for anti-botulism therapy delivery. However, in this case the cargo itself cannot be cytotoxic. For endosome-dependent cytosolic delivery to be realized, a membrane translocation/release mechanism must also to be incorporated into the delivery vehicle platform. Several well-studied bacterial toxin translocation mechanisms, including those of BoNTs, cholera toxin, diphtheria toxin, or anthrax toxins, could serve as potential inspiration for practical design of such functionality.

With regard to the cargo moiety, delivery of small drug molecules would require strategies to overcome similar difficulties as those encountered with antibody-drug conjugates, including carrier-integrity, homogeneity and drug stoichiometry by the chemical coupling/modification process. Effective cytosolic delivery of small molecule drugs other than cytotoxic agents has yet to be demonstrated. Based on the current mechanistic models of bacterial protein toxin internalization, a proteinaceous cargo would be a more logical choice. This protein could be engineered with either an inhibitory peptide or a neutralizing antibody (preferably a single-chain VHH or scFv) against the target BoNT-LC serotype, or alternatively a modifying enzyme with specificity for the targeting BoNT-LC serotype.

There has been several catalytic inactive holotoxins developed [109, 128, 132–133, 136–137], which might be suitable for use as delivery platforms through a piggyback approach. However, the major obstacle to be overcome still resides in the generation of proper fusion constructs for high-yield expression and purification of stable chimeric cargo-delivery

vehicle proteins. Another important component essential for developing successful antitoxin fusion proteins is an effective method for screening optimal linkers from a library of linker-variants, which could be used to join a desired cargo protein with a target-specific delivery vehicle protein. In the course of developing our prototype GFP-BoNT/A-HC fusion [140], several residues located in the LC and belt regions of BoNT/A were selected as potential fusion points, based on their locations in the toxin crystal structure (Fig. 4). GFP-fusion constructs were constructed at residue 233, 420, 430, 490 or 544. After a considerable amount of effort, only fusion at Leu544 yielded a stable protein product in *E. coli* (Ho and Wilson, unpublished data). We also tested a series of cleavage-resistant linkers, including DE4, DE8, DE12, and DE16, and found it is possible to introduce a linker with up to 43 amino acid residues [140]. However, further evolution of this long linker, for enhancing protein stability or other favorable characteristics inside cells, is hampered in the absence of an effective screening method, other than testing separately each individual protein.

Now that a recombinant BoNT/A-HC-based delivery vehicle can be produced in *E. coli* and can specifically deliver a prototype cargo GFP into neuronal cells *in vitro*, *ex vivo* and *in vivo* [140], it should be feasible to translate this prototype cargo-delivery vehicle into a medically relevant inhibitory cargo-delivery vehicle against botulism. Options include coupling the delivery vehicle with an inhibitory cargo such as a protease activity-neutralizing scFv or VHH antibody or a pseudosubstrate-based inhibitor against BoNT-LC activity.

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Fig. (1).

Structure of BoNT/A. Shown is a molecular graphic representation of the BoNT/A structure depicting the three functional domains, generated using Pymol (PDB 3BTA): N-terminal catalytic domain (LC), residues 1–431 (yellow); active site Zn²⁺ (red); belt region, residues 450–544 (green); membrane-translocation domain (TD), residues 545–870 (magenta); disulfide bond between Cys429, Cys453 (cyan); and a C-terminal ganglioside and protein co-receptor-binding domain (BD) consisting of two subdomains, residues 871–1091 (orange) and 1092–1295 (blue).

Fig. (2).

Proposed model of BoNT intoxication of motor neurons. Shown is a schematic diagram of the proposed mode of entry and translocation of the catalytic activity cargo into the cytosol of neuronal cells. In this model, the holoprotein is cleaved either by clostridial or cellular proteases into a 50-kDa catalytic domain (LC), which is a Zn^{2+} -dependent protease that cleaves SNARE proteins involved in synaptic vesicle exocytosis, linked by a disulfide bond to a 100-kDa HC, which mediates the binding, entry and delivery of the LC (yellow) into the cytosol of neuronal cells, where the LC catalyzes the cleavage of its cognate SNARE-protein substrate(s) to block neurotransmitter release and cause paralysis. Step 1: The C-terminal binding domain (BD) of the HC comprised of two subdomains (orange and blue) bind to surface gangliosides and/or neuronal-specific protein co-receptors (Syt-I, Syt-II, SV2A, SV2B or SV2C). Step 2: The toxin-receptor complex triggers uptake into cells via receptor-mediated endocytosis. Step 3: Acidification of the endosomes induces a conformational change in the N-terminal membrane-translocation domain (TD, magenta) of the HC that leads to its interaction with the endosomal membrane. Steps 4–6: Two alternative paths leading to translocation of the LC across the endosomal membrane that are consistent with experimental findings are shown. In path 4a–6a, the HC remains intact and the low pH induces insertion into the vesicle membrane, where with the assistance of the BD the TD forms a channel and facilitates the unfolding and funneling of the LC through the channel into the cytosol, where the LC refolds and acts on its target substrate(s). In path 4b–6b, the BD dissociates from the TD and the TD alone facilitates the unfolding and translocation of the LC across the membrane and into the cytosol.

Fig. (3). Cargo-Delivery Platform Designs. Shown are four general approaches toward cargo-delivery design: (A) Cell-penetrating peptide-based approaches. In this approach, the cargo-delivery vehicle consists of a cell-recognition domain (red) and a membrane-penetrating peptide (gray) that mediates translocation of an inhibitory cargo (green) across the cell membrane into the cytosol, intact or as a released cargo. (B) Piggybacked holotoxin-based approaches. In this approach, the cargo (green) is attached directly to the N-terminus of the intact holotoxin, consisting of the binding domain (red), translocation domain (yellow) and

catalytic domain (cyan). Here, the holotoxin mediates cytosolic delivery of the cargo in tandem with the catalytic domain of the toxin. (C) Toxin binding-domain-based endosomal delivery approaches. In this approach, the catalytic domain of the toxin has been replaced with the inhibitory cargo (green). The translocation domain (yellow) mediates delivery of cargo from acidic endosomes. (D) Retrograde transport-based approaches. In this approach, the cargo-delivery vehicle consists of a receptor-binding domain (red) that is linked to an intracellular-trafficking domain (purple), which transports the cargo through the retrograde pathway to a specialized cellular compartment, such as the ER, for cytosolic delivery of cargo (green).

Fig. (4).

Potential sites in BoNT-A-LC for constructing cargo-delivery vehicle fusion. Shown is a molecular graphic representation of the catalytic LC (cyan, yellow), belt (green) and the TD (magenta) of BoNT-A, generated from PDB3BTA using Pymol. The disulfide bridge connecting LC and HC through Cys429 and Cys453 are shown as grey spheres. Several residues that might serve as sites for fusion of a cargo with the HC are indicated by spheres (red).