

## Repurposed bacterial toxins for human therapeutics

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### ABSTRACT

Pathogenic bacterial toxins can be repurposed as therapeutics. Binary bacterial toxins are macromolecular complexes that are a current focus of therapeutic development. These proteins bind to surfaces of specific human cell populations and transport enzymes across membranes. Basic research has characterized bacterial toxin mechanisms and structure so that protein domains can be “shuffled” for a variety of applications. This approach delivers an already characterized enzyme to new cell types, specified by binding affinity. Separated protein components from holotoxins are also repurposed into drug delivery applications to form composite multifunctional drug delivery units. Enzymatic domains are used for cancer diagnosis and treatment, influence of intracellular trafficking, and for providing relief from pain, autonomic disorders, movement disorders, spasticity, and HIV. Technical challenges to this field are the immunogenicity, solubility and stability of therapeutic fusion proteins. Clinical intervention and predictive computational approaches identify, prevent, and remove known and predicted immunogenicity without a significant loss of efficacy. Unrealized medical potential exists in a wealth of bacterial diversity that may be captured by the repurposing of bacterial toxins.

**KEYWORDS:** bacterial AB exotoxins, protein engineering, human therapeutics, biologics, drug delivery.

### INTRODUCTION

Therapeutic repurposed bacterial toxins (repTox) are derived from naturally occurring proteins that

target, enter, and disrupt the biological structures and processes of cells. Many repTox are sophisticated membrane-associated proteins with high aqueous solubility, capable of targeted molecular transport to specific human cell types and intracellular locations. Treatments for cancer and neurological disorders have been the focus of several clinical trials [1-4], but only two have been approved for therapeutic use. *Clostridium botulinum* neurotoxin serotypes A and B (Botox<sup>®</sup>, Dysport<sup>®</sup>, Xeomin<sup>®</sup>, Myobloc<sup>®</sup>) can be purified directly from the microorganism and are locally administered cosmetics and therapeutics that cause neuromuscular paralysis. Engineered interleukin-2-diphtheria toxin (Ontak<sup>®</sup>) is an intravenously injected cancer therapeutic produced by recombinant biotechnology that combines the biomolecular functions of interleukin-2 and diphtheria toxin from *Corynebacterium diphtheria* to target IL-2 receptors on the cancer cell surface and gain entry into the cytoplasm to disrupt a vital protein translation factor. The potential of these functionally efficient bacterial components has not yet been fully realized in the context of human health, and may provide many new treatments and biomedical research tools [5-13]. Development of repTox into therapeutics has been enabled by advances in protein engineering and functional proteomics. This minireview considers current human therapeutic applications of repurposed bacterial toxins using protein engineering and biotechnology.

### Structure/function of bacterial toxins

Pathogenic bacteria have evolved to produce a swarm of proteins (Table 1), lipopolysaccharides and effectors to increase virulence [14, 15], but

**Table 1.** Number of bacterial exotoxin protein species that utilize known mechanisms of activity in humans.

Mechanism	Number of unique proteins produced by bacterial exotoxins [23]
Membrane-permealization-damaging [24, 25]	21
Binding-membrane components [14, 26]	11
Hemolysins [27, 28]	50
Cytolysins [29-33]	23
Metal-binding [34]	28
Proteolysis [35, 36]	32
Cytotoxins [37, 38]	42
RTX-Toxin [39, 40]	5
DNA damaging [41-43]	13
Monoglycosylation [44, 45]	4
Cytoskeletal rearrangement [46, 47]	43
Superantigen [48, 49]	19

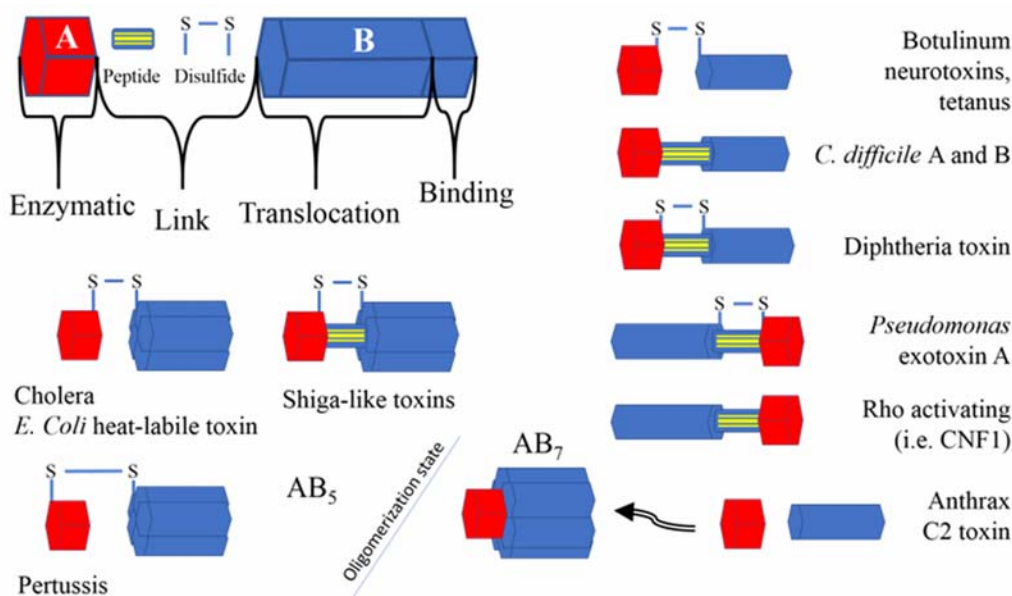
these molecules have intrinsic characteristics that can be repurposed for research and therapeutic use [16]. The most studied of these proteins in the context of therapeutic applications are exotoxins, secreted proteins that form a medically relevant basis for bacterial species and strain cladistics [17-22]. Exotoxins use a variety of mechanisms to affect their targets that are both conserved and non-conserved between bacterial species. Within species, toxins are also classified by their surface antigens as serotypes.

Of particular interest, binary bacterial exotoxins are macromolecular complexes that bind to specific cells and translocate an enzyme across a membrane to disrupt a host cell function [50-56]. Natural AB-type, or binary exotoxins, have two structural domains that contain three functional units for (1) binding, (2) translocation, and (3) intracellular enzymatic activity [57, 58]. Binding and translocation functions are commonly combined into a single domain (B) to target delivery of the enzymatic payload (A) into cells (Figure 1) [59]. This fully assembled AB-type toxin is called a holotoxin. Once the toxin binds and enters target cells by endocytosis, the payload domain is routed to an intracellular location [60, 61]. These payloads

interact directly with host cell proteins by catalysis of post-translational modifications (*e.g.*, ADP-ribosylation, glycosylation or proteolysis) or other protein-protein interactions as effectors [58].

Membrane components of human cells are molecular receptors for exotoxins, and major determinants of toxin entry and activity within a target cell [62, 63]. Many AB-type toxins typically recognize lipids and proteins displayed on the surface of target cells, whereas many pore-forming toxins require cholesterol to configure holes in the target cell plasma membrane [64, 65]. Gangliosides (glycosphingolipids) [26] are glycosylated lipids that are organized into lipid rafts on the surfaces of many cells [66-68] and normally involved in endocytosis, cell adhesion, differentiation, and growth [69, 70]. *C. botulinum* neurotoxin serotypes A and B use both gangliosides and proteins as receptors for binding and entry, a combination which enhances target cell specificity. *C. botulinum* serotypes C and D are from a genetically divergent group that have bacteriophage genetics and bind to ganglioside receptors GT1b, GD1b and GD1a [20, 71]. In the lab, receptors on the cell surface can be enriched by exogenous addition (gangliosides), choice of cell lines used in cell culture, or by genetic manipulation of those cell lines. These methods enable or increase target cell sensitivity to bacterial toxins for *in vitro* analysis. Diphtheria toxin is able to amplify its own target cell sensitivity by binding to a membrane-anchored precursor protein of heparin-binding epidermal-growth-factor-like (HBEGF) [72, 73]. HBEGF is a member of a multi-protein complex that upregulates HBEGF surface display upon ligand binding [74]. In the case of a diphtheria-bound complex, this results in higher host cell sensitivity by upregulation of HBEGF, thereby enriching the cell surface with diphtheria toxin receptors [72, 75-77].

Once bound, binary exotoxins enter the cell by specific routes of endocytosis to reach intracellular compartments. Toxins may utilize multiple mechanisms of endocytosis by the target eukaryotic cell [78] that ultimately require biomolecular machinery to provide shape and movement to internally budded membrane vesicles. Events mediated by clathrin [79, 80], caveolae/caveole-1 [61, 81, 82], GPI-anchored protein-enriched early endosomal compartments, dynamin [83, 84], and flotillin [85-89] are necessary for toxin endocytosis



**Figure 1. Structural configurations of binary exotoxins.**

AB-type toxins may be organized by functional domain orientation, stoichiometry, size, and characteristic inter-domain linkages. Cleavage of the A and B domains may be performed by proteases from the toxin-producing bacterium or the target cell to enable or increase the activity of an enzymatic payload. Structures are not drawn to scale.

and subsequent activity. Remarkably, shiga toxin from *Shigella dysenteriae* and *E. coli* shiga-like toxin binding domains can bind to membrane ganglioside Gb3 or induce their own uptake by bending target membranes into tubular entry vesicles in a clathrin and caveolin 1-independent endocytosis [90-93].

Bacterial toxins reach intracellular compartments such as endosomes, lysosomes, golgi apparatus, endoplasmic reticulum (ER), nucleus, and cytoplasm through endocytic cargo sorting and conformational changes of toxin structure [94-101]. Conformational changes in translocation domain structures initiate the formation of pores, the unfolding and threading of payloads through pores, and the refolding of payloads on the opposite side of the membrane [102, 103]. Symmetrical pore-forming structures created by combinations of subunits are conserved across toxin-producing species [104-109] and can be classified by holotoxin macrostructural stoichiometry (e.g. AB<sub>5</sub>, AB<sub>7</sub>), or by their interactions with pathogen host vascular and immune systems [110, 111]. At the cellular level, the transport of proteins within endocytic membrane vesicles are commonly directed towards the lysosome degradation pathway, presenting a barrier to drug delivery [112-114]. However, bacterial toxins can commandeer host endosome

sorting machinery to result in a routing away from the lysosome to the golgi and endoplasmic reticulum by direct molecular transport across endosome membranes (e.g. shiga, cholera, *E. coli* heat-labile toxin type I) [81, 115, 116]. Both *Clostridium* and *Bacillus* toxins such as tetanus, botulinum, difficile, iota, and anthrax take advantage of the natural acidification of endosome formation to transport a payload into the cytosol [79, 84, 117-122]. The local internal endosome pH is driven by active proton transport and results in tertiary and quaternary conformational changes of the translocation domain and payloads [123-125]. Passage of the enzyme payload through the pore is accomplished by mechanisms such as selective ion flow and Brownian ratchets [126-129]. Molecular chaperones receive unfolded payloads on the cytosolic side of the endosome membrane and aid in the reconstitution of the folded version of the payload. For example, shiga toxins are associated with Hsp40 luminal endoplasmic reticulum chaperones and associated complexes (HEDJ/ERdj3, Sec61) [130], and an essential chaperone BiP/GRP78 is required for translocation across the endoplasmic reticulum [131].

Enzymes and effectors passed through bacterial toxin pores disrupt target functions [46, 57, 132-134]. These enzymes are known to affect host cell

membrane integrity, cytoskeletal rigidity, protein translation, exocytosis, and vesicle trafficking. A single molecule of diphtheria can disrupt protein synthesis by catalyzing the transfer of ADP-ribose from NAD<sup>+</sup> to a modified histidine (diphthamide) of elongation factor-2 (EF-2) [135, 136]. The most potent bacterial toxins known are the *C. botulinum* neurotoxins, which are metalloproteases that are lethal in the nanogram per kilogram body weight range [137, 138]. Upon reaching an intracellular localization, these proteolytic enzymes inactivate SNARE proteins involved in exocytic vesicle docking and fusion, and are known to maintain activity for prolonged periods of time and to travel within or between cells [79, 139-141]. As an example of a cytoskeletal rearranging toxin, the *C. botulinum* C2 toxin is a non-covalent combination of a light chain ADP ribosyltransferase (C2I) and an activated heavy chain C2IIa heptamer that associates before endocytosis by a clathrin and Rho-dependent mechanism [84]. After an endosome pH shift, the C2IIa heptamer forms a trans-membrane pore and passes C2I into the cytosol [84]. Once in the cytosol, C2I ADP-ribosylates Arg-177 of G-actin to prevent further polymerization, causing an eventual depolymerization due to the dynamic equilibrium of F-actin [142, 143]. The C2 toxin is used to study the actin cytoskeleton because endocytosis occurs by binding to N-linked glycans on the cell surface, which is non-specific enough to result in a broad host cell sensitivity [144-146]. The preferred substrate of C2I is  $\beta/\gamma$ -actin, and its influence on cytoskeletal functions results in cellular effects such as disruption of epithelial and endothelial barrier functions or complete depolymerization of the cytoskeleton [147].

### Repurposing/engineering toxins

Knowledge of bacterial toxin mechanisms and human consequences can be used to engineer new molecules in therapeutics and research. Biomedical applications have taken advantage of cell-targeting specificity, intramolecular transport, and enzymatic activity of human disease-causing exotoxins [122, 148, 149]. Binary exotoxin domain fusions, created by recombination and joining of proteins from other toxins, human proteins, or combinatorial peptide and antibody libraries, are generated by replacement of at least one of three major functional components (binding, translocation, payload) with the intention

to deliver a characterized enzyme to new cell types or to modify existing binding affinities and kinetics [148-150]. This approach is a recapitulation of genetic recombination in the natural environment, which has resulted in the natural evolution of strains producing chimeric proteins such as the *C. botulinum* C/D mosaic toxin [151-154]. Transition of this natural approach into the laboratory with genetic engineering has resulted in artificially produced combinations of binary exotoxins. For example, the replacement of the C2 toxin binding domain with the *C. botulinum* serotype C1 binding domain specifically retargeted multiple non-toxic payloads and the native enzymatic payload C2I to neurons [149]. The combination of *E. coli* shiga-like toxin binding domain with *Pseudomonas aeruginosa* exotoxin A translocation domain-payload has been used to shrink xenograft tumors in mice using a p53 peptide-derivative payload [148]. Bacterial binding domains separated from the rest of a toxin have been repurposed into cellular binding components in drug delivery targeting applications by chemical crosslinking of binding domains to small molecules, toxins or liposomes to form multifunctional drug delivery units [114, 155-159]. A secondary toxin of *Vibrio cholerae*, the Zona Occludens toxin, binds to a surface receptor to initiate signal cascades to result in the opening of intestinal tight junctions. This activity enhances entry of cholera toxin into the bloodstream and has been repurposed for oral drug delivery applications [160, 161].

In a similar manner, other binding ligands have been combined with the enzymatic domains of toxins. Antibodies and their fragments, affibodies, or human polypeptide ligands such as interleukins are also used to change binding specificity [162, 163]. Toxins have been targeted to cancer cells using antibodies to antigens presented on the cell surface [164-167]. Similarly, affibodies [168] and small single chain proteins engineered for a binding affinity have been used for specific targeting of *Pseudomonas* exotoxin A towards HER2 cancer cells, or to achieve a 25-fold extension of *in vivo* half-life using engineered human serum albumin-binding domains from Streptococcal Protein G [169-172]. Natively structured human protein ligands applied as targeting moieties, such as interleukins, combined with diphtheria toxin enzyme have been used to treat CD-25 positive cutaneous T-cell

lymphoma using engineered binding affinity towards tumor cells that overexpress the IL-13 receptor (GB13) and IL-2 receptor (Ontak<sup>®</sup>) [173-178]. Gene therapy-based induction of cancer cells to express cytolytic agents as endogenous anti-tumor agents is an alternative approach that does not require engineered binding of a protein [179].

Although binding and enzymatic functions of repTox are a major focus of research and therapeutics, trafficking of enzymatic payloads is a salient feature. AB toxin translocation domains take advantage of early endosome pH shifts to form transmembrane pores (e.g. diphtheria, anthrax, *C. botulinum* toxins) [180-182] or utilize host protein translocation machinery after reaching the ER (e.g. shiga, cholera, pertussis) [183]. Shifts in the endosome pH alter anthrax protective antigen conformation to present a trans-membrane loop, forming a multimeric trans-membrane pore [184, 185]. Cholera toxin and *Pseudomonas* exotoxin A use ER-retention signal peptide sequences and are COPI-dependent for retrograde transport from the ER, while shiga toxins utilize multiple mechanisms [186-189]. Replacement of the KDEL-like sequence of *Pseudomonas* exotoxin A with the common KDEL sequence [190] increases toxicity by increasing utilization of a host protein ER translocation mechanism [191]. These characteristics present sequence-specific information useful for the design of repTox that may guide prediction of intracellular payload localization.

Repurposed binary exotoxin enzymatic domains are used in applications such as cancer diagnosis and treatment [192], modulation of intracellular trafficking [92], pain management [193-195], autonomic disorders [196], movement disorders [197, 198], spasticity [194, 199, 200], and HIV [201]. Understandably, potent toxins require precision use in a clinical setting to prevent side-effects [202-205]. Enzymatic payload domains can be rendered non-toxic to generate vaccines and delivery systems by simply ablating critical amino acid residues [206, 207]. Complete truncation and removal of protein sequence regions can also be used to generate smaller, non-toxic payloads for the development of delivery systems. For the *C. botulinum* C2 toxin, a minimal region of amino acid residues 1-87 of the C2I payload domain is required for non-covalent association with the C2II translocation domain,

allowing for the construction of C2I peptide fusions or chemical conjugates that utilize the translocation domain for entry [82, 83]. These truncated C2I domains have been shown by fluorescent localization and cell fractionation to provide delivery of biotinylated compounds to epithelial cells, macrophages, and Jurkat T-cells [84-86].

Immunogenicity is a major challenge for all therapeutic proteins, including repTox. The major approaches to mitigating immunogenicity are prevention and intervention [208, 209]. Anti-drug antibodies (ADA) that are produced by the human immune system in response to protein therapeutics can alter the pharmacokinetic and pharmacodynamic properties of a therapeutic by increasing drug clearance and lowering efficacy [210]. Adverse events such as anaphylaxis, cytokine release syndrome, and cross-reactive neutralization of critical patient proteins have affected therapeutic development for many proteins in clinical trials [211, 212]. To prevent ADA accumulation, proteins can be coated with polymers to sterically shield immunogenic amino acid residues from the patient immune system. These methods, such as PEGylation, PASylation, XTENylation, and reductive methylation can reduce toxicity and also improve pharmacokinetics [213-216]. *Pseudomonas* exotoxin can be selectively derivatized with polyethylene glycol (PEG) by conjugation to cysteine residues [217], or selectively glycosylated by utilization of alternative recombinant expression systems [218, 219]. Rational design and predictive computational approaches have used protein structure-based algorithms to identify and remove known and predicted regions of *Pseudomonas* exotoxin that stimulate B- and T- cell responses without significant disruption of therapeutic efficacy [220-222]. *In silico* and *in vitro* assays are used to predict immunogenicity in humans but are not yet able to displace the translational quality of a working mammalian immune system [223-227]. In preclinical trials, immunosuppression with methotrexate or tacrolimus/sirolimus reduces immunogenic risk for human therapeutics in development that exhibit higher animal immunogenicity in comparison to humans [228].

Interventions beyond molecular approaches can reduce antigen recognition such as formulations that reduce aggregation or clinical manipulation

of dose, route, and frequency [229, 230]. Dosing studies can optimize administration to prevent accumulation of ADA and adverse events while accomplishing therapeutic treatment goals. RepTox without human protein components or prolonged exposure to the immune system are less likely to develop cross-reactive antibodies, so are considered a lower immunogenic risk [231]. The clinical success of the natural botulinum toxin (Botox<sup>®</sup>) is based on its formulation, dosage, and routes of injection to avoid both immunogenicity and the consequences encountered during a natural infection [232, 233]. In response, precise methods have been developed to guide injection and to stimulate toxin endocytosis. Electrostimulation, electromyography, or ultrasound modulate cellular uptake or improve accuracy of target tissue localization by allowing medical practitioners to identify muscular injection sites by contraction or by visualization [234-236]. For example, 400 units, or ~300 nanograms of *C. botulinum* serotype A can be administered in a three month interval at multiple injection sites (intramuscular, intradetrusor, or intradermal) for use in multiple indications with rare instances of antibody production (<2%) [237, 238]. By comparison, ADA accumulation is higher with intravenous delivery of proteins containing antigens previously recognized by a patient's immune system. For example, Ontak<sup>®</sup> is used to treat cutaneous T-cell lymphoma with a 9 or 18 µg/kg/day intravenous infusion over 30 to 60 minutes for 5 consecutive days every 21 days for 8 cycles. A study showed that 66% of patients had previously been immunized with diphtheria and were already producing low antibody titers prior to therapy, and that 100% of patients developed diphtheria antibodies after a third treatment. As a result of immune responses, clearance increased two to eight fold and pharmacokinetic parameters decreased substantially. Despite immunogenicity, 30% of patients treated with Ontak<sup>®</sup> experienced a 50% reduction in tumor burden during a six week period. Seven patients (10%) achieved a complete response and 14 patients (20%) achieved a partial response [239]. Interestingly, Ontak<sup>®</sup> targeting of CD4<sup>+</sup> CD25<sup>+</sup> cutaneous T-cell leukemia/lymphoma may also counteract its own immunogenicity by also targeting healthy CD4<sup>+</sup> CD25<sup>+</sup> regulatory T-cells [240, 241]. Ontak-like molecules have been produced in alternative expression hosts or

with the goal of improving target specificity by modification of the binding domain [242, 243].

Production of repTox is not possible without overcoming challenges in cell line development and bioprocessing. Foremost, it is necessary to produce recombinant repTox in expression systems that are insensitive to the toxin. Some recombinant host expression systems such as *E. coli* and many eukaryotes such as Chinese hamster ovary (CHO) cells are sensitive to expression of certain toxin proteins, motivating the use of mutagenized expression systems and yeast strains (*e.g.* *Pichia pastoris*, *Saccharomyces cerevisiae*) [244-247]. Because mammalian cells are usually targets of toxins, production of repTox in mammalian cell lines is particularly challenging. To overcome this problem, specific cell line engineering is usually required. For example, a CHO expression system was developed for insensitivity to diphtheria and *Pseudomonas* toxins by substitution of arginine for glycine at codon 717 of EF-2 to prevent ADP-ribosylation [218]. Solubility and stability of therapeutic fusion proteins can also be major hurdles to biomolecular engineering. Many bacterial toxin fusion proteins produced in recombinant expression systems aggregate during purification or form intracellular inclusion bodies [248, 249]. Solubilization of bacterial toxin inclusion bodies with a chaotrope followed by separation of the chaotrope from solution [250] is used to refold diphtheria and *Pseudomonas* exotoxin fusion proteins with comparable structure and biological activity [251, 252]. Biophysical analysis can suggest changes in the design and formulation of a protein with respect to stability and solubility by incorporating salt effects on the protein surface charge [253], alteration of buffer ions to prevent aggregation [254], or utilize energetic equations to predict conformational equilibria [255, 256]. Protein linkers from natural and engineered sequences are often employed between functional domains to improve solubility and stability and have a wide variety of available properties such as length, flexibility, and charge [257].

## CONCLUSION

Therapeutic value can be created by repurposing the typical features of binary AB-structured bacterial toxins into drug delivery systems [258]. Clinically

and commercially available toxin entities rely upon designs that conserve natural protein features. The approach to reroute or retarget toxins by modification of binding specificity has resulted in clinical trials and FDA-approved therapies [3, 4]. Genetic engineering and shuffling of toxin protein domains between bacterial species can combine the qualities of multiple toxins in a single molecular unit. Traditionally, binding and enzymatic components of a toxin have been prioritized as fusion partners due to their localized cytotoxic activity. Immunogenicity, solubility, and stability of proteins are significant challenges, but many experimental and computational approaches exist to reduce immune responses and off-target toxicity [259, 260]. A variety of repTox are now known and progressing through clinical development for therapeutic use.

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#### CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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