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Antivirulence Agents

Targeting Bacterial Toxins

*Mattias E. Ivarsson, Jean-Christophe Leroux, and Bastien Castagner**

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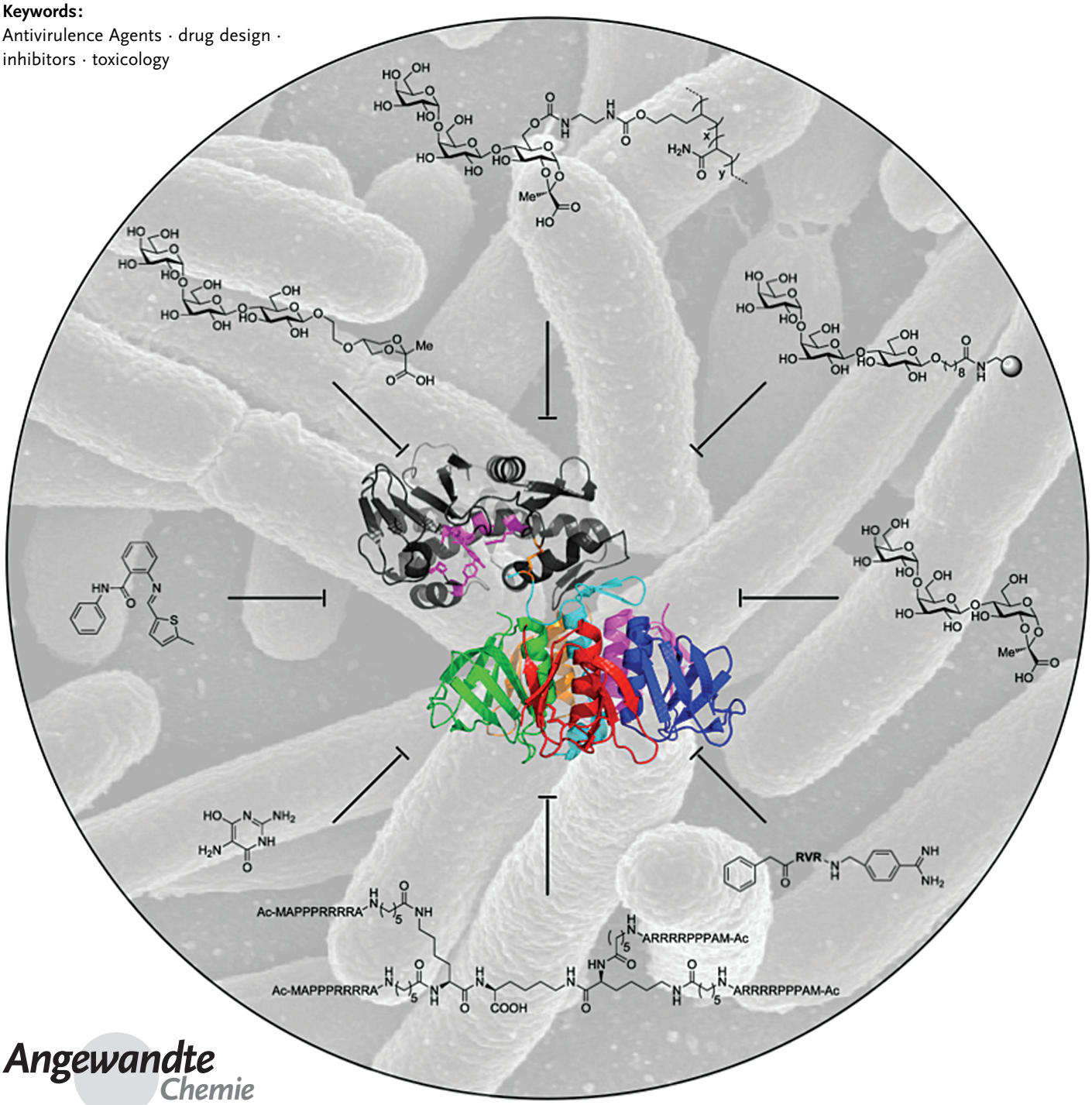


Protein toxins constitute the main virulence factors of several species of bacteria and have proven to be attractive targets for drug development. Lead candidates that target bacterial toxins range from small molecules to polymeric binders, and act at each of the multiple steps in the process of toxin-mediated pathogenicity. Despite recent and significant advances in the field, a rationally designed drug that targets toxins has yet to reach the market. This Review presents the state of the art in bacterial toxin targeted drug development with a critical consideration of achieved breakthroughs and withstanding challenges. The discussion focuses on A–B-type protein toxins secreted by four species of bacteria, namely Clostridium difficile (toxins A and B), Vibrio cholerae (cholera toxin), enterohemorrhagic Escherichia coli (Shiga toxin), and Bacillus anthracis (anthrax toxin), which are the causative agents of diseases for which treatments need to be improved.

From the Contents

1. Introduction

Protein toxins constitute an important part of the virulence factors that mediate the harmful effects of pathogenic bacteria. Since the discovery of diphtheria toxin in 1888, over 300 bacterial toxins have been identified and many of them are now recognized as being the causative agents of a multitude of bacterial diseases.[1] These include widely known diseases such as cholera and anthrax, as well as emerging threats such *as* Clostridium difficile infection (CDI).

Antibiotics have long been the preferred treatment for bacterial diseases. However, there are several advantages in targeting virulence factors rather than the bacteria themselves.[2] First and foremost, such treatments apply less evolutionary pressure on bacteria and are therefore less likely to induce the emergence of resistant strains—a problem that has been recognized as one of the most important challenges of the 21st century.[3] Second, toxins can continue to cause symptoms even after bacteria have been cleared from the host.[4] Third, non-antibiotic treatments circumvent the disruption of the normal microbiota that is typically associated with antibiotic treatments.

Bacterial toxins have an impressive variety of mechanisms of action: they can act on receptors at the cell surface, disrupt the plasma membrane, or enzymatically modify intracellular targets.[1] The latter type are known as A–B toxins, with the binding (B) moiety mediating receptor binding and toxin translocation, and the active (A) moiety containing an enzymatic domain, which disrupts cellular homeostasis.[5] The detailed understanding of these mechanisms has provided scientists with fertile ground to devise creative approaches to inhibit bacterial toxins. The steps in the molecular mechanism of pathogenicity of secreted A–B toxins are shown in Figure 1. This Review presents different chemical approaches to target these steps, provides the context to understand them, and underlines the unique challenges in translating these concepts into effective therapies. The first part of this article presents an overview of the existing knowledge about pathogenesis and current therapies for the toxin-mediated diseases caused by four well-documented species of bacteria (*Clostridium difficile*, *Vibrio cholerae*, enterohemorrhagic *Escherichia coli*, and *Bacillus anthra*cis). The second part describes selected approaches to inhibit the pathogenicity of the toxins.

1.1. Clostridium difficile (Toxins A and B)

CDI is responsible for a spectrum of nosocomial diseases ranging from mild diarrhea to life-threatening conditions such as toxic megacolon, septic shock, and intestinal perforation.[6] The three main risk factors for CDI are antibiotic treatment, hospitalization, and age.[7] Treatment with broad-spectrum antibiotics against anaerobes alters the balance of bacterial species found in the gut. This dysbiosis facilitates the

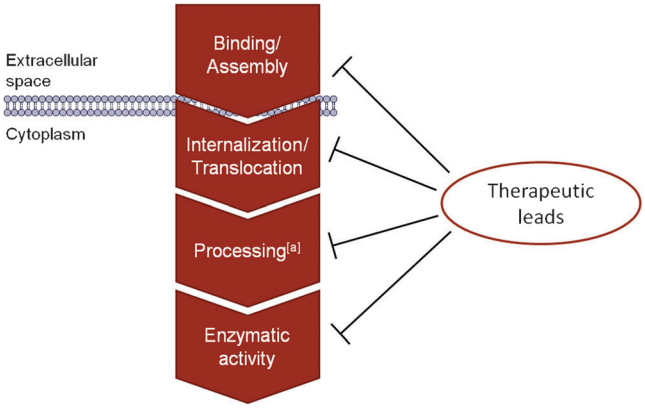


Figure 1. Schematic representation of the steps of bacterial toxin pathogenesis in eukaryotic cells, which can potentially be targeted by therapeutic molecules. Such molecules include polymeric binders, insoluble sorbents, peptides, peptide analogues, and nonpeptidic small molecules. [a] Processing can occur before internalization/translocation (e.g. anthrax toxin).

[*] M. E. Ivarsson, Prof. J.-C. Leroux, Dr. B. Castagner
Institute of Pharmaceutical Sciences
Swiss Federal Institute of Technology Zurich (ETH Zurich)
Wolfgang-Pauli-Strasse 10, Zurich (Switzerland)
E-mail: bastien.castagner@pharma.ethz.ch

colonization of the large intestine by *C. difficile*.[6] In the last 15 years, *C. difficile* has gained attention in North America and Europe because of significant increases in the frequency and severity of infection.[8] These observations have been traced back to the emergence of an epidemic, hypervirulent strain of *C. difficile* termed toxinotype III ribotype 027.[9] The increase in severity of *C. difficile* associated diseases is characterized by higher incidence of toxic megacolon, more cases in younger patients, and higher relapse and death rates.[10]

C. difficile colonizes the gut by using flagella and proteases to penetrate down to the enterocyte layer to which it adheres through adhesins.[6] There, it secretes three toxins with varying contributions to virulence: toxin A (TcdA), toxin B (TcdB), and *C. difficile* transferase (CDT).[11] CDT is a binary actin-ADP-ribosylating toxin that has been shown to induce the formation of microtubule projections in cells, but its role in *C. difficile* virulence remains unclear.[12] TcdA and TcdB are glucosyltransferases (GTs) that modify the actin cytoskeleton by glucosylating the Rho GTPases RhoA, Rac, and Cdc42 found in mammalian cells.[13] The action of both toxins on the cytoskeletal regulatory machinery causes cell rounding and ultimately cell death.[14] TcdA and TcdB are responsible for the pathogenesis; however, recent literature offers conflicting reports on which of the two is more injurious. TcdB is more important than TcdA in infection models, but strains producing only the latter are still capable of inducing disease and death in hamsters.[15]

The closely related toxins TcdA and TcdB can be divided into four functional domains: a C-terminal receptor-binding domain, a hydrophobic translocation domain, an autoprocessing cysteine protease domain (CPD), and a glucosyltransferase domain (Figure 2).[14,16] TcdA and TcdB bind enterocytes via their receptor-biding domain.[17] TcdA has been shown to bind glycoprotein96 (gp96) on the surface of human colonic epithelial cells, although details of the interaction remain unknown.[18] TcdA binds to various glycans, including the trisaccharide αGal(1-3)βGal(1-4)GlcNAc, but this sugar is not found in human tissue.[19] The characterization of the receptor for TcdB remains even more elusive despite the fact that TcdB is toxic to a broad range of cell types, suggesting that its receptor is ubiquitously expressed.[14] Once bound to the cell surface, TcdA and TcdB are internalized through endocytosis (Figure 3). Acidification of the endosomal compartment induces a conformational change that exposes the hydrophobic region in the putative pore-forming domain and

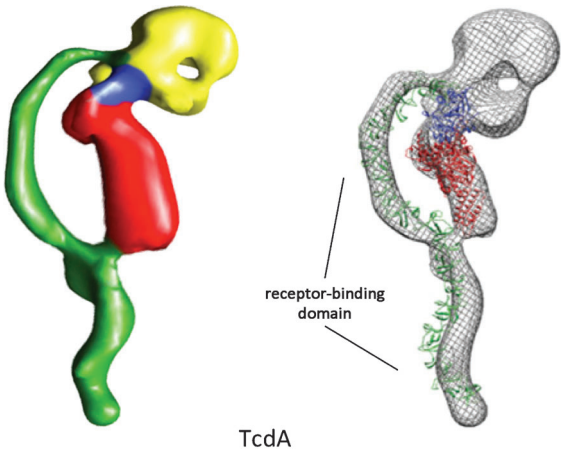


Figure 2. The structure of TcdA revealed by negative-stain electron microscopy with the glucosyltransferase domain (red), the cysteine protease (blue), the translocation domain (yellow), and the receptor-binding domain (green). The image on the right is an overlay of the image on the left with known crystal structures.[20]

leads to membrane insertion, with the N terminus protruding into the cytosol and the C terminus remaining inside the endosomal compartment.[16]

Myo-inositol hexakisphosphate (IP6), a signaling molecule ubiquitously found in the cytosol of mammalian cells, binds to the CPD after it is transferred to the cytosol, and activates the CPD resulting in autoprocessing of the toxins.[21] Toxin cleavage releases the glucosyltransferase domain into the cytosol, where it induces pathogenesis.

CDI is a significant and growing problem with at least 250000 hospitalized cases a year, a mortality rate of 1–2.5%, and an estimated cost of several hundred million dollars annually in the United States.[22] Oral vancomycin or metronidazole have proven to be the most effective treatments for CDI since the 1980s.[23] Recent studies with fidaxomicin, a narrow-spectrum antibiotic with high selectivity for *C. difficile* over other bacteria, showed cure rates similar to that of vancomycin but a reduced rate of relapse.[24] Fidaxomicin was approved by the FDA in May 2011 and could replace vancomycin as a first-line treatment for CDI.[25]

The treatment of CDI with antibiotics has several inherent drawbacks. First, there are growing concerns over the emergence of antibiotic-resistant strains that complicate treatment. Second, high recurrence rates after treatment with antibiotics have been reported.[23a] Third, an increasing

Bastien Castagner obtained a BSc in Chemistry at the University of Montreal in Canada. He then completed a PhD in Organic Synthesis at Columbia University in New York in 2004, before joining the group of Prof. P. H. Seeberger at the ETH Zurich for a postdoctoral stay. He is currently a Group Leader in the Institute of Pharmaceutical Sciences at the ETH where his research focuses on drug discovery and drug delivery.

Jean-Christophe Leroux heads the Laboratory of Drug Formulation and Delivery at the ETH Zurich. He received his PhD in Pharmaceutical Sciences in 1995 from the University of Geneva under the direction of Prof. R. Gurny and completed postdoctoral training at the University of California, San Francisco, in the research group of Prof. R. Siegel in 1997. Prior to joining the ETH in 2008, he was Professor of Pharmaceutical Technology at the University of Montreal.

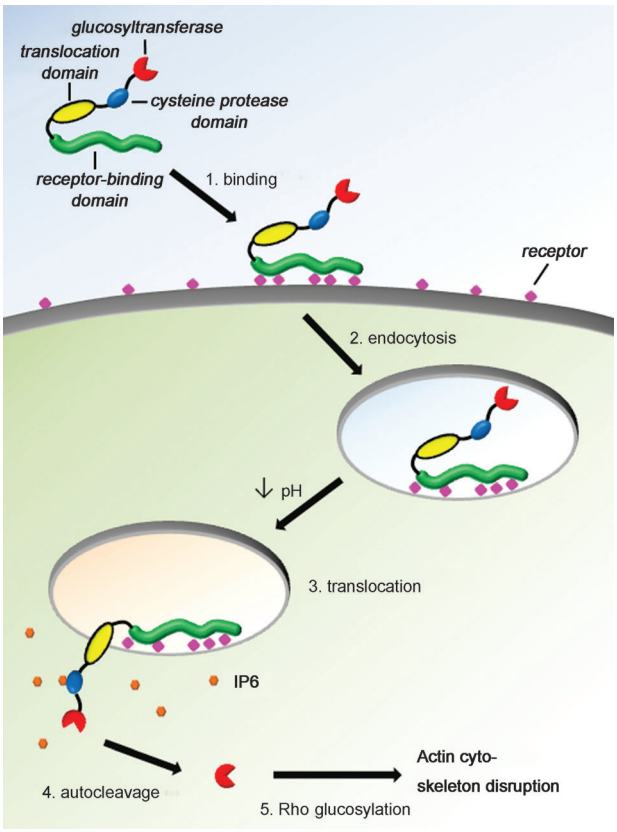


Figure 3. Molecular mechanism of action of TcdA and TcdB. The toxins bind to the surface of enterocytes through the receptor-binding domain and are endocytosed. Acidification of the endosome leads to translocation of the enzymatic domain and the CPD into the cytosol. Cytosolic IP6 then binds to the CPD, thus activating it and initiating autoprocessing. The released enzymatic domain catalyzes the transfer of a glucose moiety to a conserved threonine residue on Rho/Ras proteins, which inhibits downstream signaling events.

number of cases that are refractory to antibiotics have been reported.[26]

Developing treatments that target *C. difficile* toxins rather than the bacterium itself has the advantage of maintaining homeostasis of natural gut flora, rather than further disrupting it. This is of key importance because in individuals with a healthy gut flora, the presence of endogenous bacteria acts as a protective barrier against *C. difficile* proliferation.

Mattias Ivarsson obtained his MSc in Biomedical Engineering from the ETH Zurich under the Excellence Scholarship and Opportunity Programme in 2010. He is currently a PhD student in the group of Prof. J.-C. Leroux in the Institute of Pharmaceutical Sciences, where he focuses on developing novel therapeutic approaches for the treatment of C. difficile infection.

Strains of *C. difficile* that do not express the toxins but can compete with the pathogen are in phase II clinical trials.[27] Fully human monoclonal antibodies against TcdA and TcdB have been shown to reduce the likelihood of recurrence of CDI when co-administered with antibiotics in clinical trials.[28] In addition, a toxoid vaccine containing inactivated toxins A and B is currently in phase II clinical trials.[29]

1.2. *Vibrio cholerae* (Cholera Toxin)

Cholera is a diarrheal disease caused by *V. cholerae*, a bacteria transmitted by contaminated food and water.[30] Cholera is endemic in many regions, and the World Health Organization estimates the number of deaths attributed to cholera to be 120000 each year.[31] The symptoms are often hard to distinguish from those of other diarrheal diseases, a fact that complicates its diagnosis and reporting. Cholera symptoms include voluminous watery diarrhea and vomiting, and typically appear after an induction period of 1–5 days. The treatment is simply parenteral and/or oral rehydration. However, treatment has to be administered rapidly, since severe dehydration can lead to death within a few hours of onset. Because of this, most of the deaths caused by cholera occur in countries with limited access to healthcare. Therefore, even a successful toxin-targeted therapy would have limited impact in such settings. The large amounts of water being secreted from the small intestine also make the development of oral therapies more challenging. Prevention through sanitary measures, increased access to healthcare, and the development of new oral vaccines are the most likely initiatives to avoid deaths due to cholera.

CT is an AB_5-type toxin composed of an enzymatic A subunit and a ringlike pentameric B subunit that is involved in cellular uptake (Figure 4).[32] Individually, neither

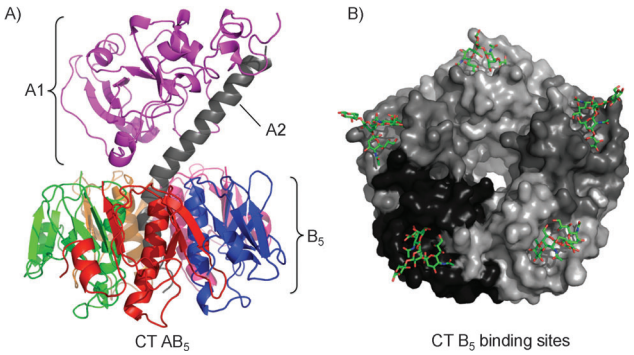


Figure 4. A) The cholera toxin AB_5 structure (PDB: 1XTC[34]). B) The B_5 subunit cocrystallized with GM1 (PDB: 3CHB[35]).

the A nor the B_5 subunits are toxic. The A subunit is divided in two parts. A1 is the enzymatic portion of the toxin responsible for the toxicity, and is linked to the pentameric B_5 subunit by the A2 domain. The A1 and A2 domains are linked by a disulfide bond.[33]

The B5 subunit binds to GM1 ganglioside on the cell surface (Figure 5).[36] After endocytosis, the whole toxin is

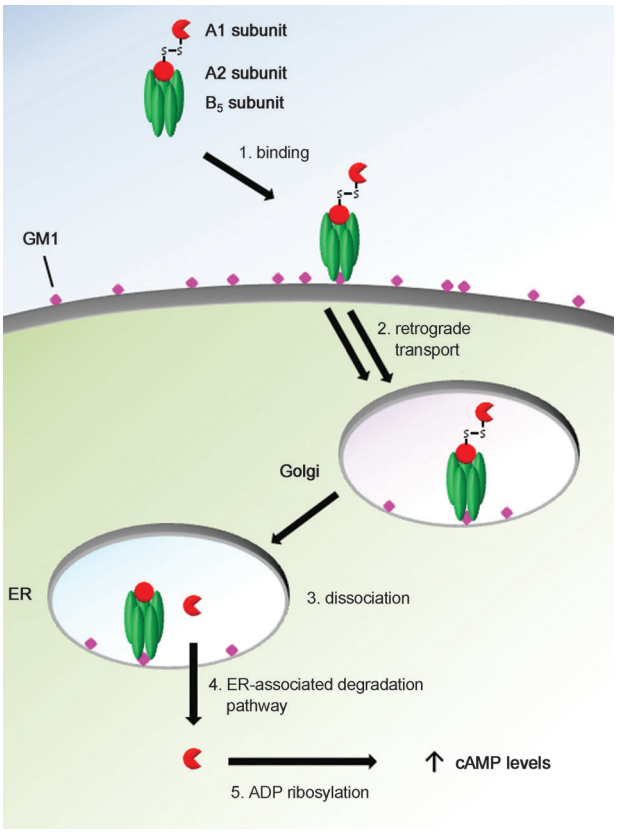


Figure 5. Molecular mechanism of action of CT. The whole toxin binds to GM1 ganglioside, followed by endocytosis. After retrograde transport via the TGN, the A1 subunit reaches the cytosol where it ADP-ribosylates the trimeric Gsα component of adenylyl cyclase.

transported to the endoplasmic reticulum (ER) by retrograde transport through the trans-Golgi network (TGN).[37] At this point the A1 domain separates from the A2–B_5 complex, unfolds, and translocates to the cytosol by hijacking the ER-associated degradation pathway.[33,38] The A1 subunit is an ADP-ribosyltransferase that targets the trimeric Gsα component of adenylyl cyclase (AC). Once ADP-ribosylated, AC remains in its active GTP-bound state, which results in increases in the concentrations of cyclic AMP (cAMP) and chloride ions, which then leads to massive fluid secretion from the small intestine.

CT has roughly 80% homology with the *E. coli* heat-labile toxin (LT) and shares the same cell-surface receptor. Enterotoxigenic *E. coli* (ETEC), which produces LT and/or the heat-stable toxin (ST) is responsible for the so-called "traveller's diarrhea" that is symptomatically indistinguishable from cholera.

Because of its lack of toxicity and high mucosal antigenicity, recombinant CT B subunit is part of the oral cholera vaccine Dukoral, along with various inactivated *V. cholerae* strains.[39] Treatment with antibodies seems unlikely in situations where basic healthcare facilities are lacking because of cost issues, and because of the harsh conditions of the gastro-intestinal (GI) tract. However, egg yolk IgY obtained from hens immunized with inactivated bacterial and CT B subunit was protective to suckling pigs against challenge with *V. cholerae*.[40] IgY from egg has the advantages of being cheaper to produce and can be lyophilized and administered orally.

1.3. Enterohemorrhagic Escherichia coli (EHEC)

Enterohemorrhagic *E. coli* (EHEC) is an important pathogen transmitted through food and water that causes severe gastroenteritis (so-called "hamburger disease").[41] It produces the phage-encoded Shiga-like toxin (SLT, also Shiga toxin or verotoxin) as its main virulence factor. Shiga toxin, the namesake of SLTs, was initially discovered in studies focused on *Shigella dysenteriae*, which produces the toxin, but the name was kept to describe the very closely related SLTs produced by *E. coli*. Stx from *S. dysenteriae* Stx and Stx1 secreted by *E. coli* are virtually identical. Stx1 and Stx2, another SLT produced by some strains of EHEC, have approximately 60% sequence homology. The main cause of outbreaks in parts of Europe and North America is usually *E. coli* O157:H7, although some recent outbreaks in Europe were caused by the O104:H4 strain.[41,42] SLTs are released by the bacteria in the lumen of the GI tract, causing diarrhea, and they then penetrate the intestinal submucosa and enter systemic circulation. This can cause acute kidney injuries and central nervous system complications, especially in children.[43] EHEC is responsible for most of the hemolytic uremic syndrome (HUS) in children in regions where the bacteria is endemic. Although HUS is a potentially fatal condition, current treatments are mostly supportive, and antibiotics are potentially damaging,[44] perhaps because of bacterial lysis or increased SLT production.[45] The usual course of an *E. coli* O157:H7 infection results in diarrhea three days after ingestion of the bacteria, followed by bloody diarrhea one to three days later.[46] This is usually when the patient seeks medical attention and a culture test reveals the presence of EHEC. The disease can then resolve spontaneously or deteriorate to HUS. The latter is characterized by microvascular thrombi and swollen endothelial kidney cells caused by circulating SLTs. By the time the presence of EHEC is confirmed, the toxin-related damage is usually well underway, making rapid intervention crucial for the success of any toxin-targeted therapy.[46] Recent literature suggests that the damage to kidney by SLTs during HUS is in part mediated by the complement system and that the monoclonal C5 antibody Eculizumab might be useful for the management of HUS in children.[47]

SLTs are AB_5-type toxins, similarly to CT (Figure 6).[48] The assembled toxin interacts with the globotriaosylceramide Gb_3[49] on the cell surface by means of its B_5 subunit, followed by endocytosis (Figure 7).[50] It is transported by means of a retrograde pathway from the endosome to the ER.[51] The A subunit is composed of two parts: the N-terminal A1 domain is the enzymatic domain that has to be released by a protease from the C-terminal A2 domain interacting with the B pentamer (Figure 6A). Proteolytic cleavage is mediated by proprotein convertases such as furin in the Golgi and endosome.[52] The reduction of a disulfide bond also has to take place to fully release the A1 subunit, which eventually

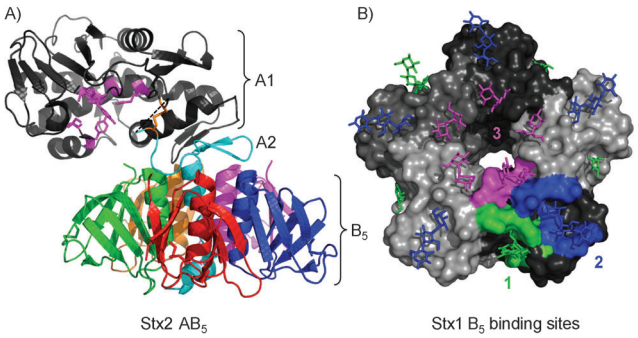


Figure 6. A) Structure of Stx2. The B pentamer is multicolored, the A2 subunit is in cyan, and the A1 subunit is in black. The enzymatic pocket is highlighted in magenta and the disulfide bond that links the A1 and A2 subunits is highlighted in orange. The loop that undergoes proteolytic cleavage is missing from the crystal structure and is represented by a dotted black line (PDB: 1R4P[34,48]). B) Binding sites on Stx1. Sites 1, 2, and 3 are highlighted on one B subunit in green, blue, and magenta, respectively, and the cocrystallized carbohydrates are showed in the respective colors (PDB: 1BOS[57]).

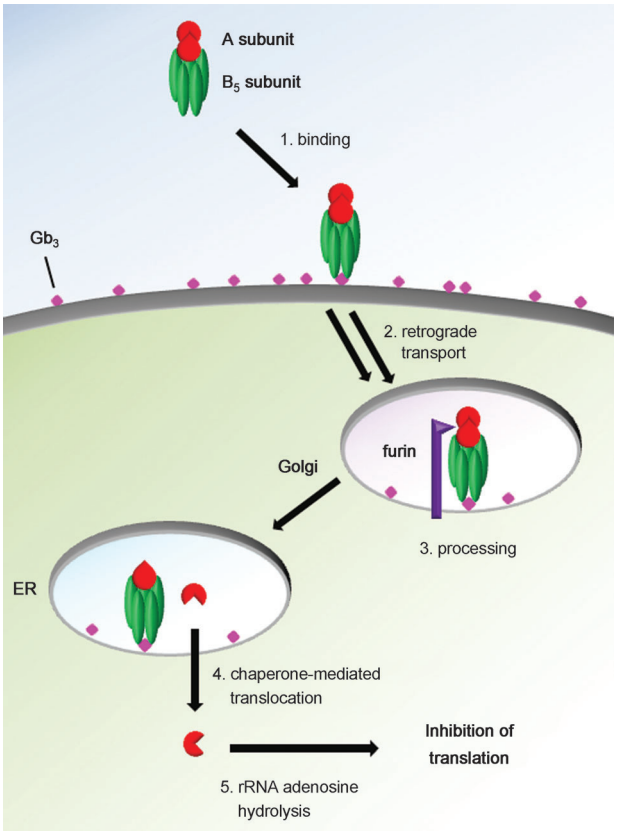


Figure 7. Molecular mechanism of action of *E. coli* SLTs. The B_5 subunit binds to the cell-surface receptor. The toxin then undergoes retrograde transport to the ER, during which it is processed by furin, liberating the enzymatic A1 subunit from the A2–B_5 complex. The A1 subunit is then translocated into the cytosol where it ultimately prevents protein synthesis.

reaches the cytosol by chaperone-mediated transfer,[53] where it acts on the 28S RNA of the 60S ribosomal subunit.[54] The A subunit of SLTs and the closely related ricin toxin (RT, *Ricinus communis*) are both *N*-glycosidases that ultimately prevent protein synthesis.[54,55] Another AB_5 toxin, subtilase cytotoxin (SubAB), secreted by *E. coli* has recently been discovered.[56] SubAB can induce pathological features similar to those of HUS in mice, but the extent of its role in HUS in the context of an EHEC infection is still unclear.

Of the two toxins, Stx2 has been linked to more severe clinical outcomes and is therefore the most important target for therapeutic interventions.[58] Stx1 and Stx2 differ slightly in their carbohydrate-binding domain. Stx2 has not been crystallized in the presence of bound carbohydrate, but Stx1 has.[57] Stx1 shows three different binding sites that seem all important in mediating cytotoxicity (Figure 6B).[59] However, in solution, the Gb_3 trisaccharide seems to bind mostly to site 2 and, to some extent to site 1, but not to site 3.[57,60] It was recently shown that Stx2 has stronger binding affinity to an *N*-acetylgalactosamine-terminated Gb_3 and that this preference was linked to site 2.[61] The bacteria can secrete SLTs in outer membrane vesicles, but the significance of this in pathogenesis is still unclear.[62]

Three humanized mouse IgG1κ antibodies against SLTs have been tested in phase I clinical studies.[63] Furthermore, human monoclonal antibodies are currently being evaluated: cαStx1 and cαStx2 (Shigamabs), directed against the Stx1B subunit and the Stx2 A subunit, respectively, and Urtoxazumab (TMA-15), directed against the Stx2B subunit.[43,64] They were all tested in healthy adults, and Urtoxazumab was also evaluated for safety in pediatric patients infected with EHEC. Both formulations were found to be well-tolerated with half-lives exceeding 9 days, so a single injection at the beginning of symptoms should hypothetically last until the onset of HUS. It remains to be seen whether these antibodies will offer protection when administered to EHEC-positive patients diagnosed after a few days of diarrhea. Another serious development problem for therapies targeting such rare and sporadic diseases is the design of a suitable clinical study with clear primary end-points, and having a population group large enough to achieve statistical significance. This is certainly a real problem for HUS, although countries where there is a higher incidence, such as Argentina, could provide large study groups.[2a] Countries in which EHEC infections are endemic could also potentially benefit from a vaccine against EHEC or its toxins. The advantage of such widespread immunization in other countries is unclear.

1.4. Bacillus anthracis (Anthrax Toxin)

Anthrax toxin is a tripartite toxin secreted by the spore-forming bacterium *Bacillus anthracis*.[65] Infection with *B. anthra*cis can be cutaneous, gastrointestinal, or pulmonary (inhalational), all of which can lead to fatal systemic anthrax disease if not treated adequately.[66] Anthrax disease in humans (inhalational anthrax in particular) has attracted significant research attention because *B. anthra*cis spores are particularly resilient and readily aerosolized, and are therefore amenable to dissemination as a biological weapon.[67]

The three proteins that constitute anthrax toxin are lethal factor (LeF), edema factor (EF), and protective antigen

(PA).[65] LeF is a zinc protease that cleaves mitogen-activated protein kinase kinases (MKKs) at the amino terminus.[68] EF is a Ca^{2+}/calmodulin-dependent adenylate cyclase that increases cytosolic cAMP concentrations to pathological concentrations.[69] PA is secreted as an 83 kDa protein that can bind either one of two cellular receptors: tumor endothelial marker 8 (TEM8 or ANTXR1) or capillary morphogenesis protein 2 (CMG2 or ANTXR2).[70] PA_{83} is cleaved to a 63 kDa protein by a protease at the cell surface or in the extracellular milieu, which allows its heptamerization into a ringlike structure (Figures 8 and 9).[71] PA_{83} is particularly sensitive to cleavage by furin and other proprotein convertases (PCs).[72] Once cleaved, bound to the cell surface, and heptamerized, PA binds LeF and/or EF, and the complex undergoes clathrin-mediated endocytosis. Acidification of the endosome leads to membrane insertion of $(PA_{63})_7$ and formation of a pore through which bound LeF and/or EF eventually translocate into the cytosol.[70,73] PA-associated LeF and EF are known as lethal toxin (LeT) and edema toxin (ET), respectively, with LeT considered the primary virulence factor.[74] None of the three proteins is toxic individually.[75]

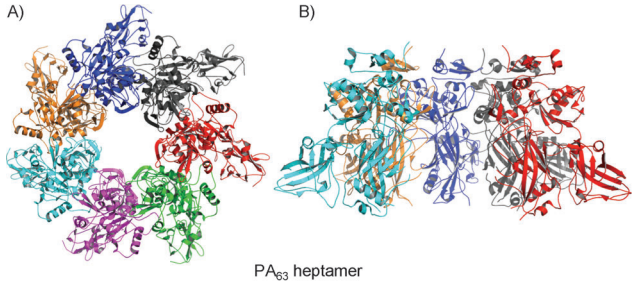


Figure 8. Crystal structure of the PA_{63} heptamer pre-pore. A) $(PA_{63})_7$ viewed from the top. B) Side view of the same heptamer. Two monomers have been removed for clarity (PDB: 1TZO[76]).

Antibiotics, vaccines, and passive immunization are all used for anthrax prophylaxis.[67] Although these approaches are effective in preventing the onset of anthrax, access to effective therapies rather than prophylactic measures would be necessary in the event of sudden and unexpected dissemination of anthrax spores. The recommended treatment for anthrax is currently limited to administration of antibiotics.[77] However, toxin accumulation in infected patients may still lead to death despite effective clearance of *B. anthra*cis. This is especially true in cases of inhalational anthrax, where disease progression is very rapid.[4b] The completion of phase III clinical trials and of animal studies in rabbits and monkeys with Raxibacumab, a human monoclonal antibody directed against PA, indicates that targeting anthrax toxins alone is sufficient to treat infection with *B. anthra*cis.[78] However, the highly malleable nature of PA implies that it can easily be engineered to become antibody-resistant.[79] This fact, together with the antibody's high cost and dosage requirements (40 mg kg^{-1}), encourages the development of non-antibody, toxin-targeted approaches to treating anthrax. The rarity of inhalational anthrax, however, poses significant hurdles in evaluating the efficacy of new therapeutics.[80]

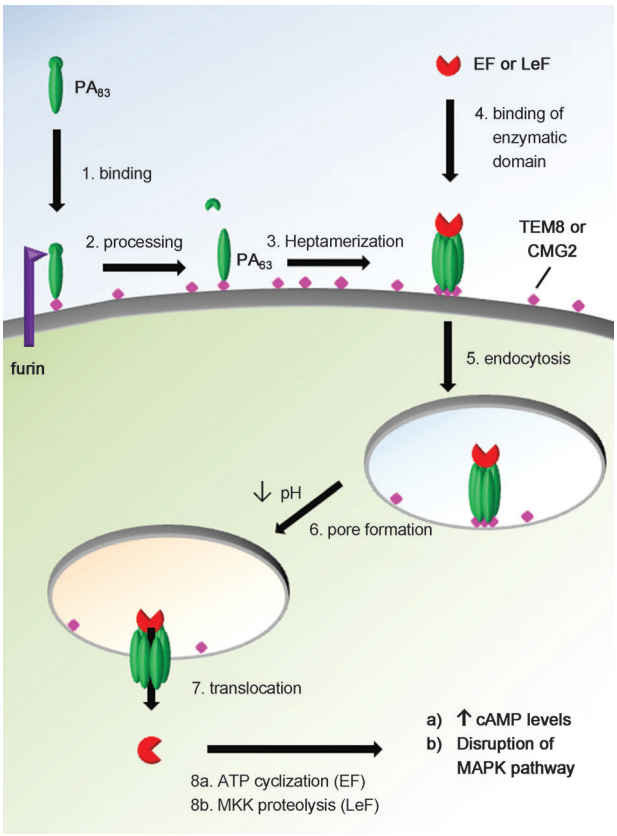


Figure 9. Molecular mechanism of action of anthrax toxin. Protective antigen 83 kDa in size (PA_{83}) binds either TEM8 or CMG2 at the cell surface and is processed to PA_{63} by PCs such as furin. PA_{63} heptamerizes, allowing it to bind EF and/or LeF. The complex formed is endocytosed and subsequent acidification of the endosome leads to translocation of the bound EF and/or LeF. Once in the cytosol, EF catalyzes the formation of cAMP from ATP and LeF mediates the proteolytic cleavage of MKKs, which disrupts the mitogen-activated protein kinase (MAPK) pathway.

2. Non-Antibiotic Therapeutic Approaches

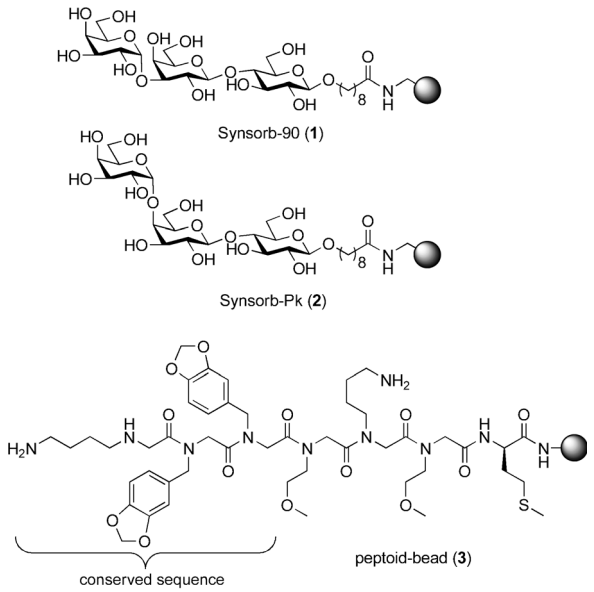
2.1. Approaches Targeting Toxin Binding/Assembly

All A–B-type toxins presented here act on intracellular proteins and therefore need to penetrate the cell membrane to cause pathogenesis. The binding of the toxin to the cell surface, prior to internalization, constitutes one of the earliest steps in the pathogenesis, which makes it an attractive target. Several approaches have been proposed to disrupt the binding of the toxin to its receptor on the cell surface, ranging from insoluble sorbents and polymers, to optimized multivalent presentations of ligand.

2.1.1. Insoluble Sorbents

For toxins released in the GI tract, insoluble sorbents can, in principle, be administered orally, bind to the toxin, and get excreted in the feces without systemic absorption. Synsorb is a sorbent that was produced by linking a toxin-binding carbohydrate ligand to Chromosorb P, a diatomite used in

chromatography. Synsorb-90, directed against *C. difficile* TcdA, and Synsorb-Pk, directed against *E. coli* SLTs, were evaluated in clinical trials but ultimately failed (**1** and **2**, respectively; Scheme 1).[81] In a phase II study, Synsorb-Pk did not diminish the severity of HUS in pediatric patients. It



Scheme 1. Structures of oral, insoluble decoys against *C. difficile* TcdA (**1**), *E. coli* Stx (**2**), and *V. cholerae* CT (**3**).

would be tempting to conclude that this study confirms that targeting SLTs in the GI tract after hospitalization is too late, but it is also possible that efficacy of Synsorb-Pk in vivo was not sufficient to generate an observable effect, perhaps because its neutralizing activity against Stx2 is lower than that against Stx1.[82] It is, however, more likely that a therapy targeting SLTs in the circulation would be more effective.

Synsorb-90 displays the trisaccharide αGal(1-3)βGal(1-4)βGlc known to bind the receptor-binding domain of TcdA and TcdB.[83] At 200 mg kg^{-1} in a mouse model, Synsorb-90 offered protection against TcdA enterotoxicity. However, at 400 mg kg^{-1}, even nonfunctionalized Chromosorb P showed activity, suggesting that the nonspecific interactions were playing a role.[84] Synsorb-90 failed in phase III clinical trial for possibly two reasons.[81b] First, it is now known that TcdB is at least as important as TcdA in causing disease whereas Synsorb-90 was only active against TcdA. This is an intriguing observation since the carbohydrate ligand should, in principle, be capable of binding to the receptor-binding domain of TcdB as well.[85] A second possible factor is the fact that the Chromosorb particles are too large to diffuse in the mucus layer that protects enterocytes, whereas *C. difficile* is known to penetrate this layer.[6,86] Presumably, a successful therapeutic agent will have to penetrate the mucus to inhibit internalization of the toxins.

In the previous examples, carbohydrates were used to obtain specific binding of the decoy to the toxin. Screening campaigns can be used to identify new peptides or peptoid moieties that bind selectively to a toxin, even if the native receptor is a carbohydrate. Peptoids are unnatural peptide analogues in which side chains are attached to the amide nitrogen on a polyglycine backbone. Since peptoids are resistant to enzymatic digestion, they constitute an attractive choice for an oral decoy. A peptoid-functionalized Tentagel bead that binds *V. cholerae* CT was identified by screening a library of up to 10^5 candidates (**3**; Scheme 1).[87] Two hits were obtained and shared a consensus sequence that was resynthesized without the last two, nonconserved residues. Beads functionalized with this consensus sequence protected intestinal cell monolayers against CT, albeit with preincubation of the beads with the toxin. Ultimately, oral therapy targeting the toxin for cholera could be complicated by voluminous diarrhea.

2.1.2. *Soluble Decoys*

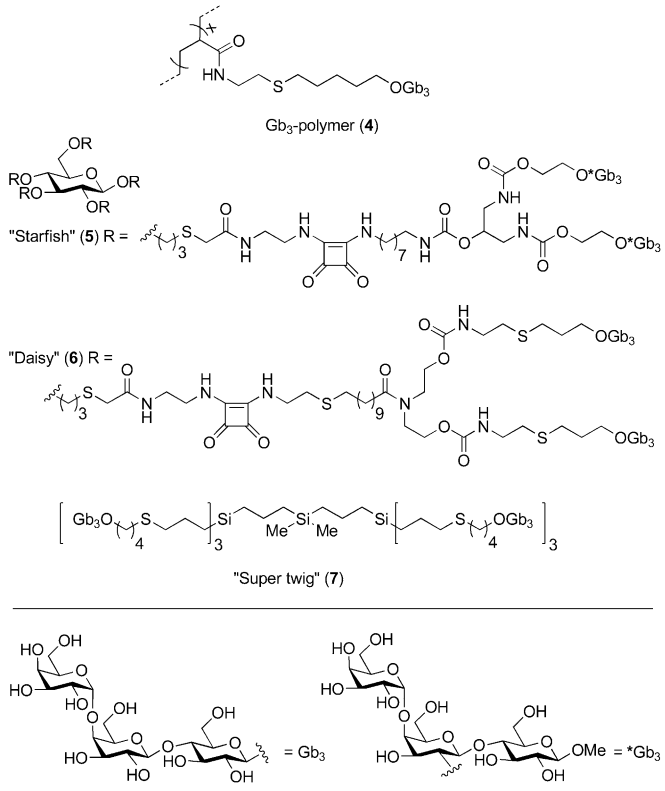
Soluble forms of native receptors involved in binding and internalization of toxins can be used as decoys. This approach was explored against *B. anthra*cis PA[88] with the advantage being that the toxin cannot be nefariously engineered to avoid binding to the receptor without influencing its activity. Soluble fragments of the PA receptors TEM8 and CMG2 were expressed in mammalian cells and tested in vitro and in vivo. The sCMG2 decoy protected rats when co-injected at or near equimolar ratio with LT. The sTEM8 was less effective in vitro and did not protect rats in the challenge experiments. The difference in activity was attributed to differences in the binding affinity of the soluble decoy and PA. Indeed, sCMG2 has a binding affinity of 170 pM for PA compared to 130 nM for sTEM8. Recently, a mutated form of sTEM8 (L56A) showed improved affinity for PA and in vivo protection similar to that of sCMG2.[89] In this study, the L56A soluble decoy showed increased plasma half-life and increased targeting to the lungs compared with sCMG2, which could explain why its in vivo efficacy is similar to that of sCMG2 despite its lower affinity to PA. The sCMG2 soluble decoy was recently evaluated in a mouse challenge using toxins engineered to be antibody resistant. As expected, the soluble decoy was still effective in protecting mice.[79] The circulation half-life of the sCMG2 decoy was increased dramatically to five days by fusing it to an antibody Fc region.[90] This approach may constitute an interesting adjunct or alternative therapy to antibody treatment, especially in the context of exposure to anthrax as a bioweapon.[89]

2.1.3. *Polymeric/Oligomeric Binders*

Polymeric binders are macromolecules that are designed to bind molecules in the GI tract and prevent their absorption.[91] These polymers are themselves not absorbed and thus generally nontoxic. For example, cholestyramine and Colesevelam are used to sequester bile acid salts and prevent their reabsorption, which results in a reduction of plasma cholesterol.[92] Other polymers are employed in the management of hyperkalemia (Kayexalate) and hyperphosphatemia (Sevelamer), and more are being evaluated, notably in the management of celiac disease.[93] Tolevamer is a polymeric binder that was designed to sequester *C. difficile* toxins.[94]

This polymer showed promising results in phase II, especially for recurrent CDI, but failed in phase III clinical trial in a non-inferiority test against metronidazole and vancomycin.[81b] The polymer had to be administered in high doses (9 g day^{-1}), perhaps because of unspecific interactions with mucins or other proteins. Tolevamer is a high-molecular-weight polystyrene sulfonate that was shown to bind TcdA and, to a lesser extent, TcdB through electrostatic interactions and multiple weak hydrophobic interactions.[95] The efficiency of the polymer to sequester TcdB was perhaps not sufficient in light of the renewed importance of TcdB in the pathogenesis.

Polymers can be grafted with recognition moieties such as carbohydrates to improve their specificity. Such glycopolymers have been used extensively to bind bacterial toxins and other virulence factors such as adhesins.[96] For example, Gb_3 trisaccharide, the receptor for SLTs, bound to a polyacrylamide backbone (**4**; Scheme 2) was shown in one report to bind both Stx1 and Stx2 and inhibit their cytotoxicity.[97] Moreover, the polymer was effective when administered intragastrically on day 3 to mice challenged with a fatal dose of *E. coli* O157:H7.[97a] However, these results contrast with that of a previous report showing that a Gb_3 polymer could bind Stx1 but not Stx2.[98] The authors suggested that the aliphatic linker used between the Gb_3 and the polymer backbone played an active role in the successful binding to Stx2, thus explaining the different results.[99] They suggested that this polymer could be a useful prophylactic agent for people who have been in contact with EHEC patients or who might have been exposed to EHEC in an outbreak.[100]



Scheme 2. Multivalent presentations of Gb_3 for SLT inhibition.

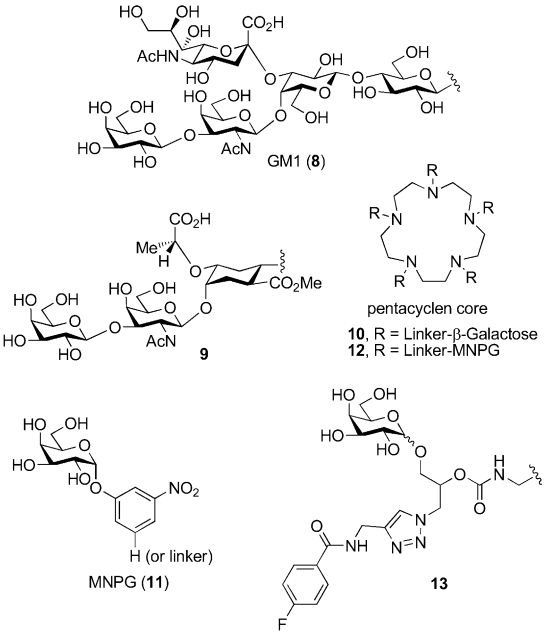
Owing to their unique pentameric structure, SLTs and CT have been the subject of very elegant studies in which multivalent displays of carbohydrates exhibited high-affinity binding to toxins. One of the most notable examples is a pentavalent display of Gb_3 trisaccharides for the binding of SLTs (Starfish (**5**); Scheme 2).[101] The design is based on a glucose core with five radiating spacers terminated by two Gb_3 trisaccharides, which are linked through their central galactose residues.[102] Starfish interacted with sub-nanomolar affinity with Stx1 and low nanomolar affinity with Stx2. The 5×2 carbohydrate design was originally planned to accommodate two binding sites on each B subunit. However, crystallography showed that Starfish was sandwiched by two B_5 pentamers, with each Gb_3 bound to only one site. Starfish and Daisy (**6**; Scheme 2), a related construct in which the Gb_3 trisaccharide is linked at its reducing end, were tested in vivo.[103] When co-injected subcutaneously with a lethal dose of either Stx1 or Stx2, Daisy was shown to offer protection, whereas Starfish was effective only against Stx1. The higher activity of Daisy against Stx2 was attributed to the flexibility of the linker, although the positioning and hydrophobic nature of the linker could also have played a role, as it was the case for the Gb_3 polyacrylamide. In a mouse model challenged with *E. coli* O91:H21 strain, daily subcutaneous doses of Daisy 24 h after inoculation with bacteria protected 50 % of the mice from death. Overall, Daisy had lower activity in vitro than Starfish but outperformed it in vivo, possibly because of different pharmacokinetic profiles. Alternatively, the biodistribution or excretion of the toxin could be different with Daisy. The authors only tracked the effect of Starfish on the biodistribution of ^{125}I-labeled Stx1 and Stx2. It was found that Starfish reduces radioactivity found in the kidney, brain, spleen, and liver for Stx1 but not Stx2.

A dendritic carbosilane core was also used to present the Gb_3 trisaccharide in a multivalent fashion.[104] The hexavalent compound nicknamed Super twig (**7**; Scheme 2) provided complete protection to mice when co-injected with a lethal dose of Stx2.[105] Importantly, Super twig was able to protect mice when injected twice daily, starting on day 3, in an *E. coli* O157:H7 infection model. Here again, the in vitro binding affinities of various constructs were not necessarily correlated with in vivo efficacy. The authors identified the ability of the complex to promote uptake and degradation by macrophages, perhaps through scavenger receptors, as being an important factor for in vivo activity. Indeed, when Super twig was co-injected with ^{125}I-labeled Stx2, the amount of radioactivity recovered from the liver and spleen was greater than that when the labeled toxin was injected alone. These examples demonstrate that in vitro binding is not always predictive of in vivo activity, and that parameters such as pharmacokinetic profile and fate of the toxin-binder complex are important as well.

More recently, a fullerene pentavalent Gb_3 glycoconjugate has been synthesized, but no binding assay to the toxins was reported.[106] The presentation of Gb_3 on surfaces or gold nanoparticles also gives good affinities that could be exploited for detection and analysis.[107]

V. cholerae CT and the closely related *E. coli* LT are both AB_5 toxins that have also been the subject of numerous

studies aimed at finding inhibitors of their interaction with the pentasaccharide GM1, their natural receptor. Dendrimers have been functionalized with GM1 (**8**; Scheme 3).[108] The multivalent display offered significant improvement over the affinity of the monomeric ligand alone. Given the complexity of the GM1 pentasaccharides, simpler moieties were explored for multivalent display. The GM1 mimic **9** (Scheme 3), in which the sialic acid is replaced by a lactic acid moiety and the branching lactose is simplified, has been presented on dendrimers and yielded more potent inhibition than GM1.[109] The terminal galactose is the most deeply buried sugar in the toxin-binding pocket, and a compound in which simple β-galactose residues radiate from a pentacyclen core (**10**) was also shown to have good binding affinity, albeit still lower than that of the GM1 natural ligand (Scheme 3).[110] The length of the linker in this case was optimized so that it would match the size of the toxin in order to have optimal binding. More recently, dendrimers decorated with four or eight galactose residues were found to have inhibition potency against CT similar to that of GM1 itself.[111] Comparable dendrimers decorated with carbohydrates have also been used on surfaces to probe multivalency effects on microarrays.[112] A small structure–activity relationship study to identify other improved ligands revealed that *m*-nitrophenyl-α-D-galactoside (MNPG (**11**); Scheme 3) was 100 times more active than galactose in a displacement assay with LT and CT.[113] While this compound is still 4 orders of magnitude away from the GM1, it constitutes a good starting point for designing a multivalent binder. The presentation of MNPG in a pentavalent fashion (**12**) was indeed accompanied by an affinity increase of 260-fold over the monomeric ligand in a displacement assay with CT.[114] However, the optimal length of the linker arm that had been previously optimized on the galactose construct **10** could not be reproduced with the MNPG because of solubility problems; the construct length was suboptimal, but **12** was active nonetheless. A later study explored the activity of "nonspanning" bivalent MNPG molecules, in which the distance between the two ligands is too short to accommodate two binding sites at the same time.[115] In this case, the improved inhibitory activity of the dimer, compared to that of its monovalent counterpart, cannot be explained by multivalency. Steric inhibition was proposed as a possible explanation. One strategy to increase the binding affinity of a carbohydrate, beyond multivalency, is to gain additional binding with a different, non-sugar moiety, as was shown by the addition of a nitrophenol group on the galactose for binding to CT and LT. A fragment-based approach was recently used where a multivalent galactose residue with a pendant azide was reacted with a library of simple alkynes by "click chemistry".[116] The resulting library was screened in a displacement assay with CT and a low nanomolar multivalent inhibitor was found (**13**; Scheme 3). The affinity of the monomeric ligand was similar to that of the monomeric MNPG.



Scheme 3. GM1 pentasaccharide LT and CT receptor and simplified analogues.

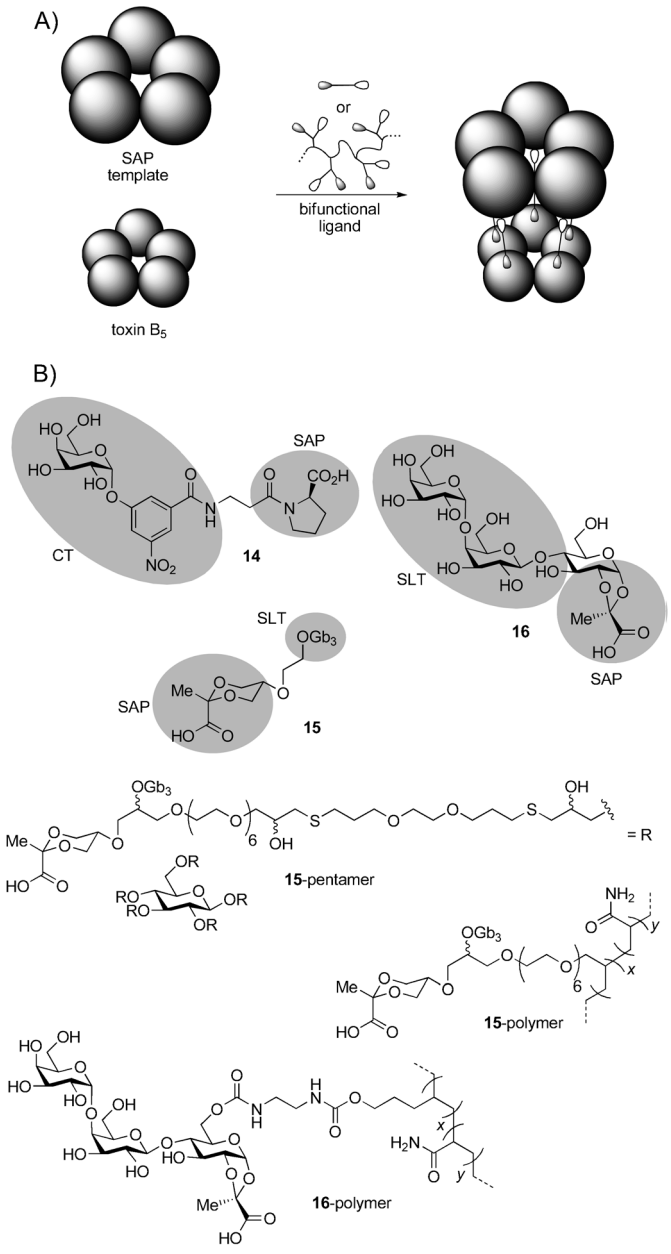
Glycopolymers with a well-defined and controlled distance between ligands are difficult to obtain if the distance is large. The only controllable parameter is the degree of functionalization of the polymer backbone, which gives a distribution of distances between ligands. Synthetic glycopeptides, in which a helical or random-coil peptide is decorated at specific amino acids with a galactose residue, were used in order to control the distance between the ligands with precision.[117] These glycopeptides displayed up to 340-fold inhibition enhancement over that of galactose in a displacement assay with CT. An optimal distance of 35 Å between the galactose residues and a helical conformation of the peptide both had positive effects on the inhibition. More recently, random-coil bivalent glycopeptides were synthesized to evaluate the influence of the charge of the peptide backbone on the binding.[118] As expected from the presence of basic residues on the protein surface surrounding the carbohydrate binding sites, negative charges on the backbone improved the inhibition of CT interaction with its receptor.

Ultimately, none of the previous molecules were tested in cell culture or in vivo, so their therapeutic potential against CT or LT is unclear. As described in Section 1.2, the binding and inactivation of CT or LT with an orally administered binder during an infection will be very challenging. The interest of these studies lies in the lessons learned in the multivalent design of potent protein binders.

An elegant solution for the controlled ordering of random polymers is to use a template to preorganize the ligand in a defined orientation. Serum amyloid P component (SAP) is a pentameric protein present in blood that was found to be the endogenous neutralizing factor for Stx2 in humans.[119] This protein circulates with concentrations of around 30 mg L^{-1} but only in humans. In fact, mice receiving twice daily injections of human SAP were completely protected against a challenge with a lethal dose of Stx2, but not against Stx1.[120] Transgenic mice expressing human SAP at levels similar to that of humans survived significantly longer than wild-type mice in the Stx2 challenge, but ultimately succumbed. The authors of this study suggested that administration of SAP could be a potential therapy for HUS. SAP itself is

a therapeutic target because of its presence in amyloid deposits. A small molecule that binds to SAP and inhibits its binding to amyloid fibrils was identified.[121] The dimerization of this molecule resulted in a ligand capable of assembling the pentavalent protein into a dimer sandwich that was quickly removed from circulation by the liver. A similar concept was applied to induce the complexation of SAP with either SLTs or CT (Scheme 4). A small molecule combining a SAP ligand and MNPG (**14**; Scheme 4) was shown to inhibit the binding of CT to its receptor and, more importantly, the presence of SAP increased the potency of **14** by almost 3 orders of magnitude.[122] A similar type of ligand was also designed for inhibition of SLTs, with Gb_3 as the SLT ligand and a pyruvate ketal as the SAP ligand (**15**; Scheme 4).[123] In this case a pentavalent presentation of bifunctional ligand **15** was also evaluated (**15**-pentamer; Scheme 4). As expected, both ligands were active in an ELISA assay, with the pentamer showing a lower IC_{50} value, and both displaying a significant decrease in IC_{50} in the presence of SAP. Perhaps not surprisingly, the effect of added SAP was more dramatic for **15** (280-fold increase in potency) than for **15**-pentamer (35-fold). Since **15**-pentamer is already preorganized into a pentamer, we do not expect significant increase in activity upon SAP templating.

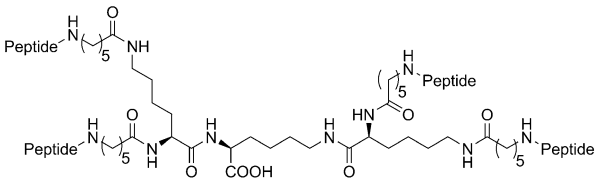
An improved bifunctional ligand design led to compound **16**, which merges the Gb_3 and the pyruvate ketal without rotatable bonds in between the two ligands.[124] This change led to a 50-fold improvement in the IC_{50} value with an enhancement of inhibition of around 4 orders of magnitude in the presence of SAP. Remarkably, this small molecule was as active as Starfish (Scheme 2) in a Vero cell cytotoxicity assay. Unfortunately, compound **16** was inactive in a mouse model because of its rapid clearance by the kidney. Acrylamide polymers functionalized with either **15** or **16** showed increased inhibition of Stx1 compared to the monomeric ligand, and a large enhancement in the presence of SAP (**15**-polymer, **16**-polymer; Scheme 4).[125] Interestingly, a polymer randomly displaying both a SAP ligand and Gb_3 did not show any improvement in inhibition in the presence of SAP. Unlike the monomeric **16**, **16**-polymer showed activity in transgenic mice expressing human SAP. The polymer has a larger hydrodynamic volume and probably exists as a complex with SAP, which would explain why its circulation time is greater than that of monomeric **16**. The polymer was able to protect 100% of mice against Stx1-induced lethality in a model where Starfish was only able to delay death. No in vivo experiments were performed with Stx2 because of its binding to human SAP in the absence of ligand. **16**-polymer was shown to influence the biodistribution of ^{125}I-Stx1, notably reducing the amount of radioactivity in the kidneys and brain. Most of the toxin appeared to be metabolized in the liver. Clarifying the precise role of SAP in the context of human infection will be crucial in determining the clinical potential of this approach.[126]



Scheme 4. A) Templating strategy where a circulating pentameric protein is used to preorganize a monomeric or polymeric bifunctional ligand to effectively bind the B_5 subunit of a toxin. B) Different bifunctional ligands for SAP-templated inhibition of CT and SLT.

2.1.4. *Multivalent Peptide Binders*

Whereas carbohydrates represent an obvious starting point in the design of molecules intended to inhibit the binding of toxins to their carbohydrate receptors, they offer certain disadvantages. First, the affinity of the monovalent ligand for the toxin is almost invariably low, thus requiring multivalency for high-affinity binders. Second, some of the more complex carbohydrates are still difficult to synthesize in large quantities. One alternative is to use peptides, which can mimic carbohydrate epitopes. Furthermore, peptides are easier to synthesize and libraries of peptides for screening can be assembled readily by solid-phase synthesis. A library of tetravalent peptides, presented on a lysine trimer, was screened for its interaction with the Stx2 B_5 subunit.[127] Peptide **17** (Scheme 5) protected mice even when given



17, Peptide = Ac-MAPPPRRRRA-

Scheme 5. Tetravalent presentation of the SLT-binding peptide.

intragastrically three days after infection with *E. coli* O157:H7. Surprisingly, the peptide did not prevent cellular uptake of the toxin to the Golgi, but disrupted the transfer to the ER. This system worked only intragastrically in mice, and not intravenously (i.v.).[128] However, when evaluated in a non-human primate toxemia model that displays a pathophysiology closer to human HUS, the tetravalent peptide **17** was effective at rescuing baboons from an otherwise lethal dose of Stx2 after i.v. dosing.[129] When **17** was administered at 5 mg kg^{-1} 24 h after challenge with Stx2, followed by a daily 1 mg kg^{-1} dose up to day 4, three out of four baboons survived, and showed protected kidney functions and preserved urine output. While closer to human HUS, this model is not perfect and does not involve an infection with bacteria. Further evaluation in an infection model will certainly be of interest for bringing such a therapy closer to the clinical stage.

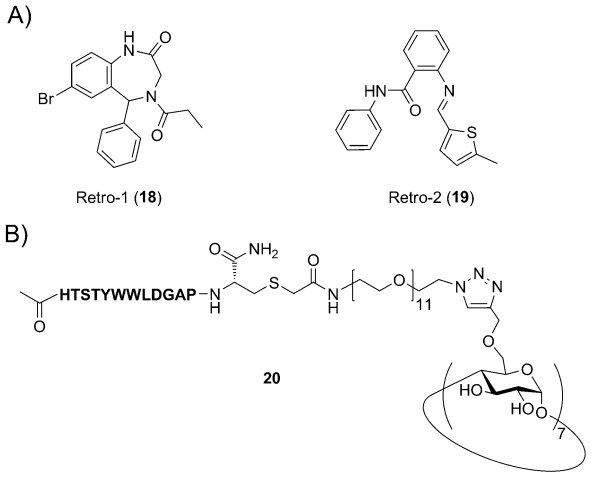
Multivalent peptides can also be used as synthetic equivalents of soluble decoys (see Section 2.1.2) to prevent protein–protein interaction between the toxin and its receptor or prevent toxin assembly. For anthrax, multivalent peptides were used to inhibit both PA–receptor and PA–LeF interactions. A phage-display library was employed to identify a dodecameric peptide that binds to PA_{63}, albeit modestly, and weakly inhibited its interaction with LeF.[130] When this peptide was conjugated to a polyacrylamide backbone, the multivalency resulted in a lowering of the IC_{50} value to 20 nM in a cytotoxicity assay, a drop of almost 4 orders of magnitude. The polymer protected rats against LeT, when injected 3–4 min after the challenge.

A similar approach was applied to identify a peptide that binds the cell-surface receptors TEM8 and CMG2.[131] A phage-display selection experiment identified a peptide that prevented association between both receptors and PA. Multivalency was achieved by decorating liposomes with multiple copies of the peptide. While this peptide was technically designed to target the receptors of the toxin, and not the toxin itself, the inhibition on cells turned out to work by steric inhibition of the LeF binding to the heptameric PA_{63}, not by inhibiting the interaction between PA and the receptor. Presumably, the peptide-binding site on the receptor does not overlap with the PA-binding site, so the binding and assembly of PA_{63} are not inhibited, but the complex is then unable to bind to the LeF. To explain the discrepancies with the phage-display results, the authors speculated that steric inhibition from the phage might have disrupted PA binding. Nevertheless, co-injection of LeT with the liposomes prevented rats from becoming moribund.

2.2. Approaches Targeting Toxin Trafficking

Once endocytosed, A–B toxins reach the cytosol by one of two possible routes: either directly from endosomes (e.g., anthrax toxin, diphtheria toxin, TcdA, TcdB) or from the ER after undergoing retrograde transport from early endosomes through the TGN (e.g., CT, SLTs).[132] The latter route of transport appears to be unique to toxin proteins, offering a potential target for inhibitory molecules.

The only molecules that have been shown to inhibit retrograde toxin trafficking in vivo to date are Retro-1 and Retro-2 (**18** and **19**, respectively; Scheme 6 A).[133] Indeed, the intraperitoneal (i.p.) injection of Retro-2 at doses as low as



Scheme 6. A) Inhibitors of the retrograde route of trafficking employed by, for example, SLTs. B) β-cyclodextrin derivative that blocks EF and LeF translocation through the PA-heptamer pore.

2 mg kg^{-1} were found to protect mice from a nasal RT challenge that was lethal to 90% of the animals after 21 days when administered 1 h prior to the toxin. Furthermore, doses as high as 400 mg kg^{-1} were not toxic to the animals. Retro-1 and Retro-2 appear to act on the cellular trafficking machinery rather than on the toxins and as such also have the potential to inhibit other toxins that rely on retrograde trafficking. The authors found that relocalization of the SNARE protein syntaxin 5 correlated with blockage of SLTs in early endosomes. A better understanding of the mechanism of action of Retro-1 and Retro-2 may reveal their actual target and allow for their further optimization.

2.3. Approaches Targeting Toxin Translocation

A common feature of A–B-type toxins is their ability to transfer an enzymatic domain to the cytosol of target cells. Translocation is the series of conformational changes that toxins undergo that result in a shift of the catalytic domain from the noncytosolic to the cytosolic side of the plasma membrane. The translocation of toxins that enter the cytosol directly from endosomes is triggered by acidification of the local environment, in contrast to the translocation of toxins

that enter the cytosol following retrograde transport, which is mediated by chaperones and translocators.[132] Indirect inhibition of A–B toxins that depend on endosome acidification for translocation can be achieved by administration of drugs that block endosomal acidification, but this does not constitute a viable method of treatment given the potential toxicity issues associated with such a therapy.[134]

The translocation of anthrax EF and LeF has been studied in particular detail and a number of therapeutic leads have been developed that block the translocation process, thereby inhibiting pathogenesis.[135] An especially successful approach has been to inhibit translocation of EF and LeF by blocking the pore channel of heptamerized PA. Indeed, β-cyclodextrin derivatives with a sevenfold symmetry that matches that of the PA pore have been shown to have in vivo efficacy in anthrax infection models.[136] The most recently reported development of this idea consists in functionalizing cyclic β-cyclodextrin with peptides that specifically bind PA using poly(ethylene glycol) (PEG) linkers (**20**; Scheme 6B).[137] The authors reported an IC_{50} value of about 10 nM for the heptavalent peptidic inhibitor, representing a significant improvement over amine-decorated β-cyclodextrin derivatives. In vivo results with the peptidic inhibitor showed that it protected rats from anthrax toxin mediated morbidity when co-injected with the toxin.

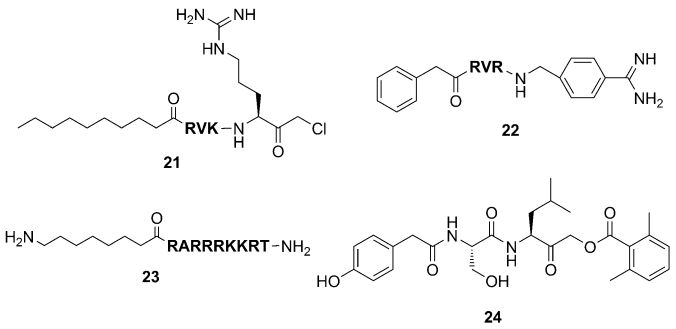
A–B toxins other than anthrax do not form stable pore structures through which the A moiety can translocate, making it impossible to target them in an analogous manner. However, translocation by means of the ER-associated degradation pathway requires an unfolding step, which can be targeted. For example, 4-phenylbutyric acid was shown to prevent the thermal unfolding, and thus the translocation of the A1 subunit of CT.[38b] This finding provides a new avenue to explore for the inhibition of toxin translocation.

2.4. Approaches Targeting Toxin Processing

2.4.1. Proprotein Convertase Inhibitors

Proprotein convertases (PCs) are a nine-member family of Ca^{2+}-dependent serine endoproteases, seven of which target multibasic motifs (furin, PC1/3, PC2, PACE4, PC4, PC5/6, PC7).[138] Found in the secretory and endocytic pathways and at the surface of mammalian cells, these PCs are involved in activating protein precursors by cleaving them at conserved recognition sites (e.g. R-X-K/R-R↓-X).[139] Furin, a 96 kDa type-I transmembrane glycoprotein, has been studied in particular detail because of its involvement in a multitude of developmental, homeostatic, and disease-related cellular processes.[140] Furin and other PCs process key molecules in diseases ranging from Alzheimer's disease to cancer and viral infections, as well as bacterial infections. Key examples of toxins that PCs activate include anthrax toxin, SLTs, and diphtheria toxin (DT, *C. diphtheriae*). Controlled inhibition of furin and other PCs has been explored as a possible approach to limit the progression of diseases caused by these toxins.[141] Anthrax toxin in particular is an attractive target for such an approach because it is processed by PCs at the cell surface (or in the extracellular space), in contrast to SLTs and DT, whose processing is intracellular.[71] These approaches will be considered here only briefly, as they target toxins indirectly.

The PC inhibitors that have shown the most promise to date are short peptides (and their analogues). The crystal structure of furin covalently bound to the potent PC inhibitor decanoyl-RVKR-chloromethylketone (**21**; $K_i = 1$ nM; Scheme 7) has greatly contributed to the design of novel



Scheme 7. Small-molecule inhibitors of autoprocessing and PC-mediated processing of toxins.

inhibitory peptides by revealing the key features of the active site cleft.[142] An approach that has recently shown promise in further increasing the stability of peptidic inhibitors was to replace the P1 arginine of **21** by a decarboxylated peptidomimetic residue such as 4-amidinobenzylamide (**22**; $K_i = 0.8$ nM; Scheme 7).[143] This reduces the residue's susceptibility to degradation by carboxypeptidases and enables tighter binding with the target enzyme's active cleft. The lack of a C-terminal ketone group also makes the molecule less prone to racemization and nucleophilic attack.

A comparison of the residues of the catalytic cleft subsites based on homology modeling of the seven PCs reveals that the S1 to S5[*] subsites are highly conserved.[141a] This implies that peptides must be at least six residues long for them to show any degree of selectivity in binding to PCs. Recently developed peptidic PC inhibitors are based on the extended cleavage motif of a viral substrate, the avian influenza H5N1 hemagglutinin.[144] The nine-residue RARRRKKRT peptide with an N-terminal 8-aminooctanoyl group (**23**; $K_i = 8$ nM; Scheme 7) was found to reduce fatality in an inhalational anthrax model in mice at low dosage (5 mg kg^{-1} i.p.). Molecule **23** was not toxic, possibly because it selectively inhibited furin, thus allowing for endogenous processing by other PCs required for homeostasis.

[*] A nomenclature commonly used to describe the sequence of peptide residues relative to the cleavage site of protease substrates denotes the amino acid N-terminal to the cleavage site as P1. Subsequent residues are named P2, P3, and so on; the amino acids that are C-terminal to the cleavage site are denoted P1′, P2′, and so on. The corresponding protease binding sites are denoted with an S rather than a P.

All therapeutic approaches based on PC inhibitors share a common challenge: managing the functional redundancy of the PC family in processing both pathogenic and endogenous substrates. It also remains to be seen to what extent inhibition of PCs can hinder the pathogenicity of toxins other than anthrax such as SLTs, where penetration of the active molecule into the cell is required for inhibition.

2.4.2. Autoprocessing Inhibitors

TcdA and TcdB from *C. difficile* also require processing to release the enzymatic domain into the cytosol after endocytosis but rather than undergoing cleavage by PCs, TcdA and TcdB both autocleave themselves through the action of their own CPD.[21a] A similar CPD is also found in the *V. cholerae* MARTX toxin.[145] A peptide derivative has recently been designed to covalently inhibit the TcdB CPD (**24**; Scheme 7).[146] The dipeptide contains the same amino acids as those in the P1 and P2 positions of the CPD's natural cleavage site and the authors observed that adding a bulky hydrophobic group at the N terminus increased the potency of inhibitors relative to analogues having a smaller acetyl group. Interestingly, tripeptide inhibitors were less potent than the dipeptides. Molecular docking studies suggest that minimal interactions exist between the inhibitor's peptide backbone and the TcdB CPD, which implies that the development of nonpeptidic inhibitors that bind the CPD in a similar fashion to **24** is feasible. Given the similarity of the TcdB cleavage site to that of TcdA, it is conceivable that **24** also inhibits TcdA autocleavage.

An alternative approach to inhibit TcdA and TcdB autoprocessing is to induce S-nitrosylation of the active-site cysteine of the toxins, a process believed to be an endogenous mechanism of host protection from the toxins.[147] The S-nitrosylation of TcdB by *S*-nitrosoglutathione (GSNO), an endogenous source of nitric oxide, inhibits IP6-mediated cleavage of TcdB in vitro. The therapeutic potential of this route of inhibition was confirmed in a mouse model of CDI in which oral treatment with 10 mg kg^{-1} GSNO significantly increased probability of survival. Administration of GSNO intracecally and co-administration with 0.25 mg kg^{-1} IP6 resulted in a further significant increase in the protective effect. However, it is unclear to what extent GSNO may be degraded in the GI tract, or to what extent it may interact with other proteins in the GI tract.

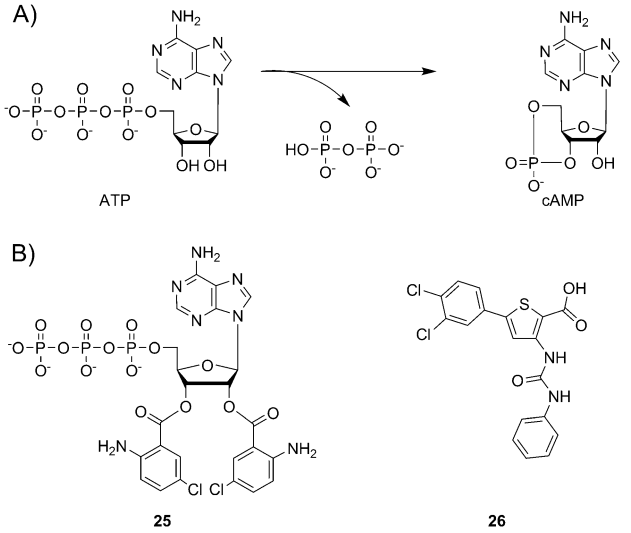
Small-molecule leads for the inactivation of TcdA and TcdB before they are internalized are particularly attractive because as the molecules must be active only in the lumen of the GI tract, membrane permeability is not a must. Furthermore, high concentrations of active compound in the GI tract can be reached.

2.5. Approaches Targeting Toxin Enzymatic Activity

Another approach to inhibit the action of bacterial toxins is to target the ultimate causative agent of cellular disruption, that is to say the toxins' enzymatic "warhead". Bacterial toxins can be grouped according to the cellular targets and/or the mechanism of action of their enzymatic domains. The enzymatic domains presented in this section are not exhaustive; examples of enzymatic warheads found in toxins that are either not important virulence factors or not A–B-type toxins (and are therefore outside of the scope of this Review) are phospholipases, deamidases, proteases (other than metalloproteases), and deoxyribonucleases.[1]

2.5.1. Adenylyl Cyclase Inhibitors

Class II adenylyl cyclase (AC) toxins (also called adenylate cyclase toxins) include EF (*B. anthra*cis) and adenylate cyclase toxin (CyaA, *B. pertussis*).[148] ACs catalyze the formation of cAMP from ATP (Scheme 8 A).[149] Since cAMP is an important second messenger, an increase in its



Scheme 8. A) Mechanism of action of adenylyl cyclases. B) Inhibitors of toxin adenylyl cyclases.

cytosolic concentration beyond physiological levels has a variety of potentially harmful effects that include increased ion fluxes and fluid secretion, which can cause edema and diarrhea, and impairment of phagocytic functions.[75,150] Ca^{2+} and calmodulin (CaM) modulate the AC activity of EF and CyaA.[151]

The inhibitors of the enzymatic activity of AC toxins developed to date inhibit either the binding of substrate (ATP) to the active pocket (competitive inhibitors) or the activation by CaM (noncompetitive inhibitors).[152] The crystal structure of EF with and without CaM has been extensively used to guide the design of EF inhibitors, and the structural homology that EF's AC domain shares with CyaA implies that inhibitors specifically designed to target EF's AC binding pocket are also likely to inhibit CyaA.[69b,153]

Anthraniloyl-substituted nucleotides display inhibitory activity against bacterial ACs.[154] One compound in particular, bis-Cl-ANT-ATP (**25**; Scheme 8 B) combines very high potency ($K_i = 16$ nM) against CyaA with 100- to 150-fold lower activity against mammalian ACs.[155] Bis-substitution of nucleotides resulted in a large improvement in the selectivity

of inhibitors for CyaA over mACs compared to that of monosubstituted nucleotides because bulky groups are more readily accommodated in the larger CyaA substrate-binding pocket.

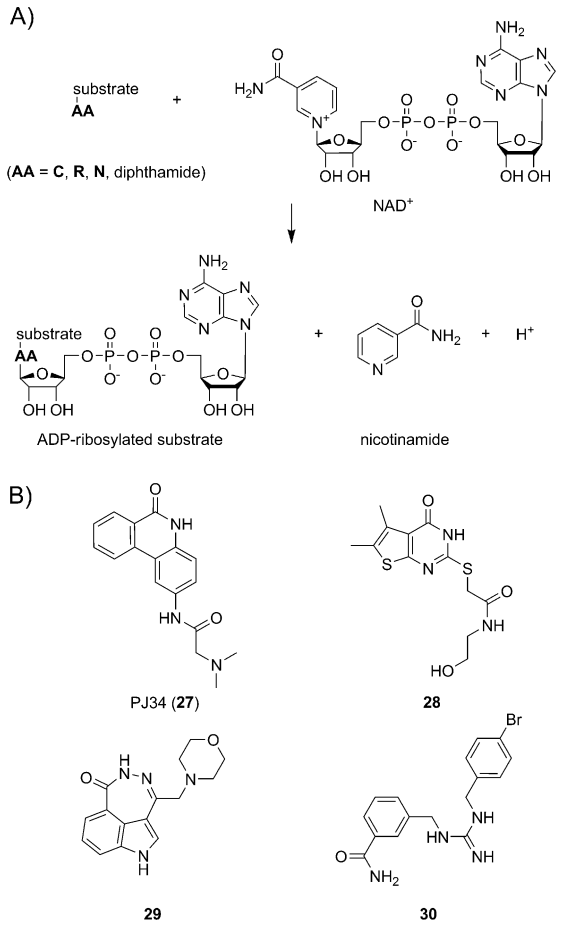
Another compound that has recently been found to inhibit activation of EF and CyaA by CaM ($IC_{50} = 2\ \mu M$ for EF) is thiophen uredoacil dichloride (**26**; Scheme 8B).[152b] The screening approach used to identify this compound involved constructing a plausible conformational path between EF's inactive and CaM-activated states in order to identify a putative pocket that could serve as a binding site for an inhibitor of activation by CaM. Inhibitors targeting binding pockets other than the ATP-binding site of AC toxins have the advantage that they are likely to be more specific and less toxic than competitive inhibitors, given the fact that ATP is a common substrate for a multitude of enzymes.

A possible reason for the lack of in vivo validation of inhibitors of AC toxins is that they are always secreted along with other toxins that may be more important for virulence. In the case of anthrax, for example, only very high doses of ET are lethal to mice (roughly $2\ mg\,kg^{-1}$) in comparison to LeT (approximately $50\ \mu g\,kg^{-1}$).[151c,156] Consequently, AC toxin inhibitors would appear to have greater potential as adjunct therapeutics rather than as standalone curative agents.

2.5.2. ADP-Ribosyltransferase Inhibitors

ADP-ribosyltransferase (ADPRT) toxins are a group of roughly 20 toxins that have mono-ADP-ribosyltransferase enzymatic activity but are otherwise not structurally related.[157] ADPRT toxins bind NAD^+, from which the ADP-ribose moiety is transferred to an acceptor protein (Scheme 9A).[158] DT (*C. diphtheriae*), CT (*V. cholerae*), LT (*E. coli*), exotoxin-A (*P. aeruginosa*), and pertussis toxin (PT, *B. pertussis*) are noteworthy examples of ADPRT toxins.[159] Although ADP-ribosylation modulates the functions of a host of endogenous eukaryotic enzymes, ADPRT toxins target only a small subset of them: actin and members of the G-protein family including heterotrimeric G-proteins (e.g. G_s and G_i), eukaryotic elongation factor 2 (eEF2), and Ras proteins (e.g. Rho family of GTPases). DT, exotoxin-A, and cholix toxin (*V. cholerae*) comprise a distinct subset of ADPRT toxins in that they ADP-ribosylate a modified amino acid residue (diphthamide) on their common target (eEF2), a property that can be exploited to tune inhibitor selectivity.[160] ADPRT toxins can be particularly cytotoxic; a classic example is that one molecule of DT fragment containing the ADPRT domain is sufficient to kill a cell.[161] Although the enzymatic domains of ADPRT toxins are attractive therapeutic targets, only a limited number of lead candidates with therapeutic potential have been identified so far.

The small molecule PJ34 (**27**; Scheme 9B) is a nonspecific competitive inhibitor of *P. aeruginosa* exotoxin-A ADPRT activity.[162] Although PJ34 inhibits exotoxin-A potently ($K_i =$ 140 nM), it is also an inhibitor of mammalian poly(ADP-ribose) polymerases (PARPs) in mouse models of stroke and therefore likely lacks the specificity to ADPRT toxins required for therapeutic use. The crystal structure of PJ34



Scheme 9. A) Mechanism of action of ADPRTs. B) Inhibitors of toxin ADPRTs.

bound to cholix toxin has recently been used as a template to carry out virtual screening with the aim of identifying novel inhibitors of ADPRT toxins.[160,163] The screen included a large library of druglike commercial compounds and a much smaller library of known PARP inhibitors. Two compounds, **28** and **29** (Scheme 9B), were found to combine high activity against cholix toxin and ExoA in cell-based assays with low toxicity and could form the basis for efforts to rationally design inhibitors with improved binding affinity. A bisubstrate analogue incorporating benzamide and guanidine moieties that mimic substrate NAD^+ and arginine, respectively (**30**; Scheme 9B), was found to have a K_i of 8 μM against CT but it remains to be seen how this compound performs in cellular assays.[164]

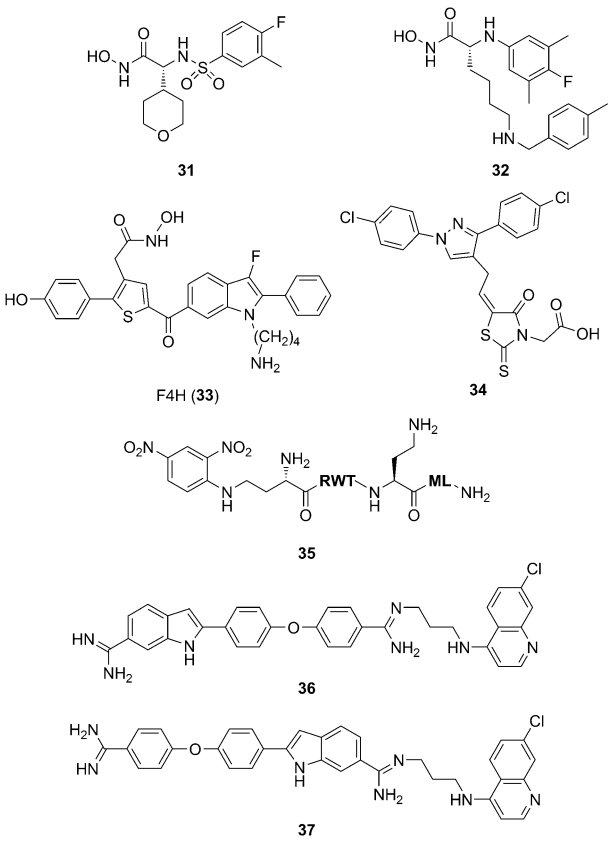
Designing inhibitors that selectively target the ADPRT domain of toxins is a challenge because despite the limited sequence identity between them and endogenous ADPRTs, they display similar structures in the NAD^+-binding region designated the "scorpion" motif.[165] Interaction with less conserved residues beyond the scorpion motif is likely necessary for molecules to discriminate between endogenous ADPRTs and ADPRT toxins. Novel fluorescence-based assays for high-throughput screening of lead compounds should contribute to the expansion of the currently limited number of ADPRT toxin inhibitors.[166]

2.5.3. *Metalloprotease Inhibitors*

Bacteria secrete a large and diverse number of zinc metalloproteases but only a handful of these are classified as A–B-type toxins (*Clostridium tetani* neurotoxin (TeNT) and *Clostridium botulinum* neurotoxins (BoNTs), *Bacillus anthracis* LeT and *Bacteroides fragilis* fragilysin).[1,167] It is worth noting that the two most potent bacterial toxins known, the clostridial neurotoxins (CNTs) BoNT serotype A (BoNT/A) and TeNT, are metalloproteases.[156,168] Recombinant *B. anthracis* producing LeF with a catalytic point mutation in the metalloprotease site does not kill mice, suggesting that inhibiting metalloprotease activity is a promising therapeutic approach for anthrax treatment.[169] Anthrax LeT and BoNTs (both Category A bioterrorism agents) are the metalloprotease toxins that have been the most popular targets for inhibitor design in recent years and are the focus of this section.[170] Metalloprotease toxins share a strictly conserved zinc-binding HEXXH motif but do not share a high degree of sequence homology outside of the zinc-binding motif, except in the case of CNTs.[167a,171] In fact, the CNTs BoNT/B and TeNT have over 50% sequence identity, and CNTs all target proteins composing the neuronal SNARE complex by similar mechanisms, ultimately inhibiting acetylcholine release at neuromuscular junctions.[172] Although *C. botulinum* produces seven different serotypes of BoNT (named A–G), only four of these (A, B, E, and F) are causative agents of botulism disease in humans. Efforts to design inhibitors of BoNTs tend to focus on BoNT/A because it is the most potent and has the longest half-life of neurotransmitter release inhibition ($t_{1/2}$ > 31 days).[173]

Molecules containing a metal-chelating hydroxamic acid group have proven to be particularly efficient inhibitors of the metalloprotease activity of BoNTs and LeF.[170a,173a] The sulfonamide-based hydroxamate **31** (Scheme 10) protected all rabbits tested in a so-called "point of no return" model when co-administered subcutaneously with ciprofloxacin, whereas ciprofloxacin alone protected 50% of rabbits from death.[4b] Compound **31** binds the LeF metalloprotease domain competitively (K_i = 24 nM); the hydroxamic acid moiety interacts with the zinc ion through a bidentate interaction.[4b] The binding pocket of LeF at the S1′ site is smaller and tighter than that of matrix metalloproteases and accommodates the aromatic moiety of **31** snuggly, conferring it with selectivity for LeF over matrix metalloproteases and several other endogenous proteases and protease-like enzymes.[174] Pharmacokinetic parameters for **31** have also been determined in several preclinical species and the results suggest that it could even be administered orally. Recent efforts have investigated structural modifications to **31** that confer it with increased in vivo efficacy.[175] Optimization resulted in compound **32** (Scheme 10) which has a significantly improved K_i (0.24 nM) that protected all rats tested from death after LeT challenge at doses of 2.5 mg kg^{-1}.[175b]

Hydroxamates have also yielded encouraging in vivo results as inhibitors of the metalloprotease activity of BoNTs. A computer-aided optimization using a cation dummy atom (CaDA) approach was used to design a micromolar inhibitor of BoNT/A, F4H (**33**; Scheme 10).[176] Intraperitoneal pre-



Scheme 10. Inhibitors of metalloprotease toxins.

treatment with F4H 30 min before the toxin challenge protected 100% of mice from death during 12 h after being challenged with BoNT/A, while 40% of untreated mice survived during the same time lapse.[176b] F4H also has a relatively long half-life in mice ($t_{1/2}$ = 6.5 h). However, it is unclear whether F4H protects mice from BoNT/A by binding extracellular toxin or by acting on the toxin directly in intoxicated neurons.[176b] It also remains to be shown to what extent F4H may inhibit endogenous matrix metalloproteases, although the molecule does not appear to elicit any acute toxicity in vivo.

The use of rhodanine derivatives as inhibitors of LeF and BoNT/A has also been explored.[177] A pyrazole derivative of rhodanine (**34**; Scheme 10) has been shown to inhibit BoNT/A and LeF competitively in the low-micromolar range and the compound increased survival of mice infected with *B. anthra*cis Sterne spores with treatment starting 24 h after exposure. However, it must be noted that rhodanines have been observed to give unusually frequent hits in high-throughput screens because of their high reactivity with proteins and interference in photometric assays.[178] The potential of rhodanines as therapeutic molecules therefore needs to be validated with rigorous assaying of selectivity and toxicity.

Peptide-like compounds modeled on residues around the scissile bond of the BoNT/A substrate (SNAP-25) have also been shown to inhibit bacterial metalloproteases in vitro (e.g. **35**; Scheme 10).[179] The insights into the conformation and chemical contacts with the target gained from structural

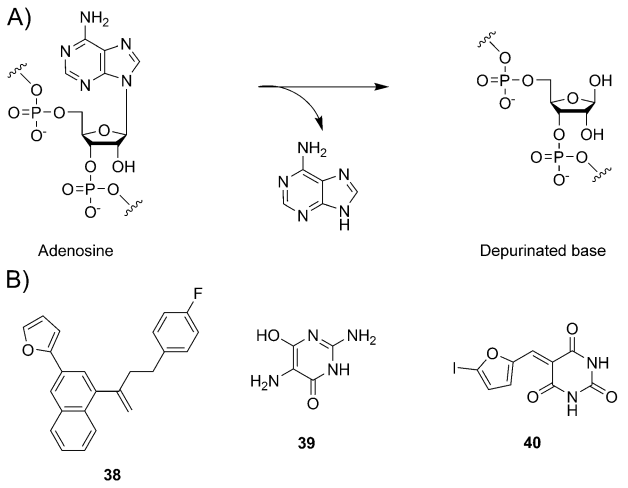
studies can be used to mine databases containing virtual small-molecule libraries with the aim of finding compounds that combine the potency of peptide-like compounds with the druglike qualities of nonpeptidic small molecules.[4a] An example of leads optimized in this fashion are the regioisomers **36** and **37** (Scheme 10), the first non-zinc-coordinating nonpeptidic small-molecule inhibitors of BoNT/A active in the sub-micromolar range in vitro.[180]

A systematic study of the effect of peptide length on inhibitory activity against BoNT/A has recently demonstrated that a simple tetrapeptide is sufficient to competitively inhibit BoNT/A with high potency.[181] The tetrapeptide RRGC (K_i = 158 nM) mimics the first four residues of the endogenous substrate, QRATKM, and therefore acts in a truly competitive fashion. The tetrapeptide was shown to be specific to BoNT/A and inhibited BoNT/A-mediated cleavage of SNAP-25 in multiple assays using chick motor neurons and primary rat and mouse cerebral neurons at micromolar concentrations. Furthermore, the roughly 500 Da tetrapeptide displayed favorable druglike characteristics such as high solubility in water, resistance to intracellular proteases, high cell penetration due to its cationic character, and low toxicity (at 600 μM), making it a promising therapeutic lead.

2.5.4. RNA N-Glycosidase Inhibitors

Shiga toxins and SLTs produced by *S. dysenteriae* and *E. coli*, respectively, are the most thoroughly studied bacterial toxins having RNA *N*-glycosidase activity.[55,182] They are ribosome-inhibiting proteins (RIPs) that act by cleaving an adenine group in a highly conserved sequence of 28S ribosomal RNA, thereby inhibiting elongation factor 1 dependent aminoacyl-tRNA binding to ribosomes and ultimately inhibiting protein translation (Scheme 11 A).[55,183] The mode of action is very similar to that of the structurally closely related RT.[184]

The challenge in developing competitive inhibitors of RNA *N*-glycosidase toxin activity is that mimicking the endogenous substrate, adenine, yields molecules that lack the solubility and potency required for therapeutic use. Furthermore, the active site of RNA *N*-glycosidase toxins is very large, making it a difficult target for drug development.[185] A recent study showed that the most potent inhibitor of Stx1A1 and RT in vitro found to date (**38**; $IC_{50} \approx 30$ μM; Scheme 11 B) was only moderately active in cell-based assays, an observation that could be attributed to the compound's poor solubility.[186] This molecule acts in a manner similar to **39** (Scheme 11 B), another inhibitor of Stx1A1 and RT, in that it interacts with its target when the enzyme is in a closed or inactive conformation.[187] Binding in the closed conformation offers a more limited surface for specific interactions than binding in the open conformation, in which adenine is endogenously accommodated.[185] Virtual screening approaches that exploit structural differences between open and closed conformations of SLTs and RT have recently been used to identify novel inhibitors that were subsequently tested in cellular assays.[185] One of the most potent compounds identified, **40** ($IC_{50} \approx 200$ μM; Scheme 11), inhibited RT activity in a cellular assay but failed to inhibit Stx1A1 and was cytotoxic at concentrations above 30 μM.



Scheme 11. A) Mechanism of action of RNA N-glycosidases. B) Inhibitors of toxin RNA N-glycosidases.

The authors of a study using a similar approach for lead validation warn that luciferase assays sometimes used for cell-free screening of lead compounds can result in false positives because of the similarity in structure between leads and D-luciferin, the substrate for firefly luciferase.[188] The development of more robust high-throughput screens for RNA *N*-glycosidase toxin inhibitors could hence contribute to the discovery of new leads. Clearly, there is a trade-off between maximizing inhibitor–target interactions to increase affinity and obtaining a druglike, small-molecule inhibitor of RNA *N*-glycosidase toxins but the aforementioned leads provide a basis for further developments.

3. Summary and Outlook

Despite the unquestionable success of antibiotics in treating bacterial infections, effective therapies are plagued by a concomitant increase in resistance against antibiotics, and decrease in number of new drugs reaching the market. Furthermore, antibiotics are not always effective against toxin-producing bacteria such as EHEC and *V. cholerae*. The value of targeting bacterial toxins has long been recognized since the early days of toxin antiserum administration and the more recent success of antibodies and vaccines. Nevertheless, there has been a renewed interest in the field stemming from the knowledge gained in the mechanisms of action of bacterial toxins. So far, the targeting of toxins has yielded important knowledge on the interaction of molecules with proteins, in particular with respect to multivalency. However, several challenges remain to be addressed if any of these strategies are to progress further towards therapies.

In academic research, the drug-delivery aspects such as permeability, solubility, and elimination are too often ignored when new molecules are designed as potential therapeutics. The lack of in vitro/in vivo correlation of activity often observed for bacterial toxin binders is a reminder that the

ultimate fate of the drug–toxin complex has to be considered. For instance, actively targeting clearance pathway such as the macrophage scavenger receptors might constitute an interesting line of research. In the case of molecules designed to bind the toxins in the GI tract, excretion is not an issue, but the harsh environment and the physical barrier of the mucus layer that protects enterocytes should be taken into account early on in the design of the drug.

Some classes of enzymes are inherently difficult targets. For example, while the glucosyltransferase domains of *C. difficile* TcdA and TcdB are viable targets, the design of inhibitors is challenging because mimicking the UDP-glucose substrate yields molecules with poor druglike characteristics. Therefore, a solution to the general problem of developing glycosyltransferase inhibitors could yield new candidates against TcdA and TcdB. The use of peptides to bind to the active site may constitute a breakthrough in this field.[189] Similarly, RNA *N*-glycosidases are attractive but challenging targets. In the case of ADPRT toxins, the main difficulty is to obtain selectivity over endogenous ADPRTs. The continued increase in throughput and reduction in false-positives in the enzymatic assays used for inhibitor screening will play a key role in the discovery of potent inhibitors of toxin enzymatic moieties.

In addition, as it has been alluded to throughout this Review, not all toxins are clinically sound targets. For example, EF from *B. anthrac*is is probably not as important as LeF in causing disease symptoms and death. Similarly, we now know that TcdB of *C. difficile* cannot be ignored since it is as least as important as TcdA in the pathology. The socio-economic context of the disease also requires consideration. Although science should ideally be free of such constraints, it is clear that a complex and expensive therapy against cholera is unlikely to make an impact in the face of simpler and cheaper alternatives like rehydration therapy. Similarly, elaborate syntheses pose a significant hurdle for commercial development of complex macromolecules, even for diseases affecting the developed world.

Recent failures of advanced candidates demonstrate that it is also of crucial importance to consider the timing and location of toxin release when considering therapeutic opportunities. For instance, targeting SLTs for the treatment of HUS is challenging in part because the therapeutic window of opportunity is very small.

We anticipate that new mechanistic insights into the mode of action of toxins will open the door for novel and more effective therapies. For instance, identification of the actual receptors for *C. difficile* TcdA and TcdB could provide useful information for the design of inhibitors. Chemists are in a position to contribute to new understanding by designing chemical tools, and to exploit this knowledge to devise innovative strategies against these diseases. The myriad of toxins produced by bacteria, coupled with our increasing understanding of their pathogenesis, should provide ample grounds for academic research to thrive.

Abbreviations

AC	adenylyl cyclase
ADP	adenosinediphosphate
ADPRT	ADP-ribosyltransferase
BoNT	botulinum neurotoxin
CaM	calmodulin
CDI	*C. difficile* infection
CDT	*C. difficile* transferase
CMG2	capillary morphogenesis protein 2
CNT	clostridial neurotoxin
CPD	cysteine protease domain
CT	cholera toxin
CyaA	adenylate cyclase toxin
DT	diphtheria toxin
eEF2	eukaryotic elongation factor 2
EF	edema factor
EHEC	enterohemorrhagic *E. coli*
ET	edema toxin
ETEC	enterotoxigenic *E. coli*
GI	gastro-intestinal
GT	glucosyltransferase
HUS	hemolytic uremic syndrome
i.p.	intraperitoneal
i.v.	intravenous
LeF	lethal factor
LeT	lethal toxin
LT	heat-labile toxin
MKK	mitogen-activated protein kinase kinase
MNPG	*m*-nitrophenyl-α-D-galactoside
PA	protective antigen
PARP	poly(ADP-ribose) polymerase
PC	proprotein convertase
RT	ricin toxin
SAP	serum amyloid P component
SLT	Shiga-like toxin
Stx	Shiga toxin
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
TEM8	tumor endothelial marker 8
TeNT	tetanus neurotoxin
TGN	trans-Golgi network

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