Arttu Laisi

EXOTOXIN TARGETED DRUG MODALITIES

Syventävien opintojen kirjallinen työ

Maaliskuu 2021

Arttu Laisi

EXOTOXIN TARGETED DRUG MODALITIES

Biolääketieteen laitos

Maaliskuu 2021

Ohjaaja: Arto Pulliainen

TURUN YLIOPISTO
Lääketieteellinen tiedekunta

LAISI, ARTTU: Exotoxin targeted drug modalities

Syventävien opintojen kirjallinen työ, 49 s.
Lääketieteellinen mikrobiologia ja immunologia
Maaliskuu 2021

According to World Health Organization (WHO), antimicrobial resistance is one of the major global health issues to track in 2021. As the efficiency of current antibiotics have gradually been declining for several decades due to the deteriorating resistance status, the demands to develop new potential antimicrobial drugs have increased rapidly. Bacterial virulence factors are molecules that enhances the probability of the pathogen to cause disease in a host. With antivirulence drugs, bacteria are not killed, but specifically disarmed by neutralizing their virulence factors, thus exposing pathogens to the influence of immunological defense mechanisms. In use of pathogen specific antivirulence drugs, the selective pressure for resistance is believed to be reduced since the drugs don't directly have an effect on bacterial viability.

Exotoxins are an extensive group of bacterial proteins, which can damage the host cells by disrupting physiological cellular functions, or directly destroy host cells, e.g. via cell lysis. Exotoxins have a significant role in bacterial pathogenicity and in some infectious diseases, e.g. cholera, tetanus and botulism, bacterial exotoxins act as the primary disease-causing virulence factor and are therefore ideal targets for antivirulence drugs.

In this review article, we focus on drug modalities, which target bacterial exotoxins. We describe how the different drug modalities work and review the key pre-clinical and clinical trial data that has led to the approval of currently used exotoxin-targeted drugs: Raxibacumab (Abthrax®), obiltoxaximab (Anthim®) and bezlotoxumab (Zinplava®). We also go through the advantages and disadvantages of these modalities and highlight the recent outcomes from preclinical and clinical trials of potential exotoxin-targeting drug molecules. The manuscript of this review article has been sent to be peer reviewed and published in *ACS Infectious Diseases*.

Key words: exotoxin, bacteria, antimicrobial resistance, antivirulence therapy, antibiotics

Exotoxin-targeted drug modalities

Moona Sakari[a,b], Arttu Laisi[a], & Arto T. Pulliainen[a*]

[a]Institute of Biomedicine, Research Unit for Infection and Immunity, University of Turku, Turku, FINLAND

[b]Turku Doctoral Programme of Molecular Medicine (TuDMM), University of Turku, Turku, FINLAND

[*]Corresponding author

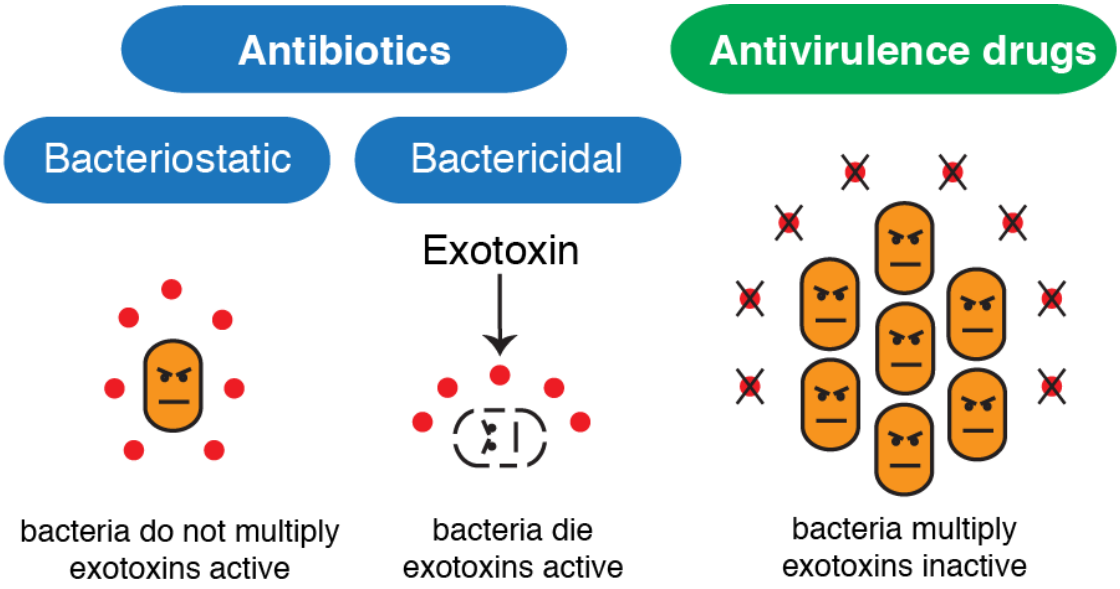
Corresponding author information:
Dr. Arto Pulliainen, Ph.D.
Institute of Biomedicine, Research Unit for Infection and Immunity, University of Turku, Kiinamyllynkatu 10, FI-20520, Turku, Finland
Phone: +358-40-1586044, Fax: not available, E-mail: arto.pulliainen@utu.fi
https://orcid.org/0000-0002-9361-8963

KEY WORDS: exotoxin, bacteria, antivirulence therapy, antibiotics, antimicrobial resistance

ABSTRACT

The paradigm of antivirulence therapy dictates that bacterial pathogens are specifically disarmed but not killed by neutralizing their virulence factors. Clearance of the invading pathogen by the immune system is promoted. As compared to traditional antibiotics, the pathogen-selective antivirulence drugs hold promise to minimize collateral damage to the beneficial microbiome. Also, selective pressure for resistance is expected to be lower because bacterial viability is not directly affected. Antivirulence drugs are being developed for stand-alone prophylactic and therapeutic treatments, but also for combinatorial use with antibiotics. This review focuses on drug modalities, which target exotoxins - a ubiquitous group of secreted or released-upon-lysis bacterial proteins. Exotoxins have a significant and sometimes the primary role as the disease-causing virulence factor. We describe the key pre-clinical and clinical trial data that has led to the approval of currently used exotoxin-targeted drugs, naimly the monoclonal antibodies Bezlotoxumab (toxin B/TcdB, *Clostridioides difficile*), Raxibacumab (anthrax toxin, *Bacillus anthracis*) and Obiltoxaximab (anthrax toxin, *Bacillus anthracis*), but also to challenges with some of the promising leads, e.g. ASN-100 (a-toxin and 5 leukocidins, *Staphylococcus aureus*) and Shigamabs (Shiga toxins 1 and 2, *Escherichia coli*). We also highlight the recent developments in pre-clinical research sector to develop exotoxin-targeted drug modalities, i.e. monoclonal antibodies, antibody fragments, antibody mimetics, receptor analogs and neutralizing scaffolds, dominant negative mutants and small molecules. We discuss how these modalities work and highlight their advantages and disadvantages as antibiotic alternatives.

TOC - Table of Content Graphic



INTRODUCTION

Bacterial virulence, i.e. ability of the bacterium to infect the host and to cause damage, is a multifactorial process involving components both from the invading bacterium and the host. The pathogen-host interplay culminates, typically in a bacterium-specific manner, into development of disease symptoms ranging from acute life-threatening conditions to chronic persistence that variably burdens the host. As defined by Diard and Hardt [1], bacterial virulence factor "is any genetic attribute that increases the chance to cause disease in a host". It is experimentally defined by the so-called molecular Koch's postulates: i) a virulence factor is expressed by the disease-causing strains, ii) deletion of the virulence factor reduces the risk of damage to the host, and iii) complementation of the virulence factor deletion mutant should restore the virulence of the pathogen.

The paradigm of antivirulence therapy, as coined in the seminal review article in 2007 [2], dictates that bacterial pathogens are specifically disarmed but not killed by neutralizing their virulence factors. Clearance of the invading pathogen by the immune system is promoted. Although the antivirulence therapy is frequently debated as a new and emerging approach, historically it precedes the use of antibiotics. The first Nobel Prize in Medicine in 1901 was awarded to Emil von Behring for his work on serum therapy, especially on its application against diphtheria with diphtheria toxin-neutralizing horse antiserum. To some extent, these virulence factor-neutralizing polyvalent antiserum-based therapeutics are still being used today, e.g. Diphtheria Anti-Toxin (DAT) [3], Botulism Antitoxin Heptavalent [A,B,C,D,E,F,G]-[EQUINE] (BAT) [4] and Botulism Immune Globulin Intravenous (BIG-IV / BabyBIG) [5]. In addition, intravenous immunoglobulin (IVIG) preparations that are composed of polyvalent immunoglobulins from pooled plasma samples of thousands of individuals are being developed and used to treat severe diseases such as necrotising soft tissue infections, e.g. [6] [NCT01790698, NCT02111161]. However, decades of basic research using various *in vitro* assays, cell and tissue culture models and animal experimentation have created an in-depth view on bacterial virulence factors [1]. It is this molecular and physiological knowledge that is driving the development of next generation targeted antivirulence therapies involving different modalities, not only antibodies. As already discussed in the seminal review article in 2007 [2], collateral damage to the beneficial microbiome is expected to be minimal along with the reduced probability to develop resistance. Antivirulence therapeutics hold promise to tackle the ever increasing problem of antimicrobial resistance, either via the combinatorial use with antibiotics or as stand-alone prophylactic or therapeutic drugs.

Exotoxins - a ubiquitous group of secreted or released-upon-lysis bacterial proteins (Figure 1) - have a significant and sometimes the primary role as the disease-causing virulence factor, e.g. in whooping cough, cholera, diphtheria, tetanus, botulism, anthrax and toxic shock syndrome.

Exotoxins are therefore ideal targets for antivirulence drugs. Exotoxins can be classified into three types based on their mode of action - Type I) superantigens, Type II) membrane-disrupting toxins, and Type III) intracellular-targeting toxins. Superantigens, such as toxic shock syndrome toxin-1 (TSST-1) of *Staphylococcus aureus* [7], bind simultaneously to major histocompatibility complex (MHC) class II and T-cell receptor (TCR) molecules on host antigen-presenting cells (APCs) and T-lymphocytes, respectively. Docking of TSST-1 to MHCII/TCR hyperactivates T-cells leading to systemic release of inflammatory cytokines and development of potentially fatal toxic shock syndrome [7]. Membrane-disrupting toxins, come in three different flavors. The pore-forming toxins, such as the a-toxin (Hla, Hemolysin-a) of *S. aureus* [7], comprise by far the largest group. When a-toxin of *S. aureus* binds on the host cell surface, it oligomerizes and attacks the cell membrane by extrusion of a b-barrel through the lipid bilayer to form a hydrophilic transmembrane channel and cell death via osmotic lysis [7]. Membrane-disrupting toxins can also act by directly modifying the membrane lipids or by displaying detergent-like functions. The b-toxin (b-hemolysin) of *S. aureus* [7], for instance, cleaves sphingomyelin, the most abundant eukaryotic membrane sphingolipid. The amphipathic peptides known as phenol-soluble modulins, such as δ-toxin of *S. aureus* [7], integrate into the host cell plasma membrane to cause membrane instability. Intracellular-targeting toxins are diverse group of virulence factors formed of either covalently or non-covalently bound A and B subunits. The A subunit possesses the enzymatic activity, and the B subunit mediates the cell entry. Pertussis toxin (PTX), as an example, is the major virulence factor of *Bordetella pertussis* [8], secreted from the bacteria via the Sec-pathway and the Ptl type IV secretion system [9]. PTX is composed of five non-covalently bound subunits (PtxS1-S5), which are arranged in an AB5-topology [10, 11]. The B5-oligomer is formed by the PtxS2-S5 (PtxS2, PtxS3, PtxS5 and 2 copies of PtxS4) [10, 11] and mediates binding of the AB5 holotoxin on the host cell surface in a carbohydrate-dependent manner [11]. Endocytosis-mediated cell entry is followed by retrograde trafficking into the endoplasmic reticulum (ER) [12], dissociation of the B_5-assembly from the PtxS1-subunit [13], which belongs to the family of ADP-ribosyltransferases (ARTs) [14], and ER-associated degradation (ERAD) pathway-dependent transport of PtxS1 into the cytosol [15]. In the cytosol, PtxS1 ADP-ribosylates a single C-terminal cysteine residue in inhibitory α-subunits of most heterotrimeric (αβγ) G protein superfamily members, such as Gαi, Gαo, and Gαt [16-18]. The resulting bulky ADP-ribose modification disrupts inhibitory α-subunit interaction with G protein-coupled receptors (GPCRs), preventing formation of

the Gαβγ-GPCR complex and thereby perturbing GPCR agonist-induced signaling [19]. Other intracellular-targeting toxins follow more or less the same principles as PTX in how they interact with the host cell, i.e. docking into the cell surface receptor, endocytosis, intracellular maturation and execution of the cytosolic activity, mostly involving modification of a specific cytosolic host protein. However, topologies of the AB-assembly vary, e.g. AB (diphtheria toxin), AB5 (pertussis toxin) and A2B5 (typhoid toxin), some toxins such as diphtheria toxin gain access into the cytosol from the endosome and an array of enzyme activities in addition to protein ADP-ribosylation are executed in the cytosol (Figure 1).

Antivirulence drugs are being developed and used to prevent all four main steps in the functional pathway of exotoxins - secretion, cell surface binding, intracellular maturation and cytosolic effector functions (Figure 1). There is active research to develop inhibitors targeting the Sec-pathway responsible for the secretion of majority of bacterial proteins, in particular the bacteria-specific SecA protein, e.g. [20]. Also, other bacterial secretion systems such as the type III secretion system (T3SS), which is responsible for the delivery of effector proteins directly into the host cell cytosol involving a needle-like apparatus, is targeted in drug development projects, e.g. [21]. Although T3SS does not classify as a *bona fide* exotoxin-delivery apparatus, it provides a proof-of-principle case on the potential of secretion inhibitors as drug leads. Phase II trial (NCT02696902) was just recently finished on the use of MEDI3902 to treat pneumonia cause by *Pseudomonas aeruginosa*. MEDI3902 is a human monoclonal antibody (mAb) that has been engineered to bind both the PcrV T3SS needle tip protein and the bacterial Psl exopolysaccharide [22]. Phase II trial (NCT01695343) has also been conducted in cystic fibrosis on a different PcrV-targeting human antibody fragment, the anti-PcrV PEGylated-Fab KB001A [23]. A modest forced expiratory volume in 1 sec (FEV1) and reduction in sputum IL-8 were recorded, but the overall efficacy of KB001A in cystic fibrosis was weak [23]. Another active line of research is focused on targeting host cell components, in particular proteins, that are important in the functional pathway of exotoxins. For instance, small molecules have been identified, which affect the endosomal maturation [24], retrograde trafficking [25, 26], intracellular activatory proteolytic processing [27, 28] and intracellular chaperon-assisted activatory folding [29]. However, a rationally designed host molecule-targeted drug that affects the functional pathway of exotoxins has yet to reach clinical trials.

This review is focused on drug modalities, which specifically target exotoxins after the secretion or release-upon-lysis, i.e. antibodies, antibody fragments, antibody mimetics, receptor analogs and

neutralizing scaffolds, dominant negative mutants and small molecules. We describe how these modalities work and highlight their advantages and disadvantages as antibiotic alternatives. Each modality is described with examples. We also provide a resource, i.e. primary research articles published on exotoxin-targeted drug modalities in the past 5 years (Suppl. Table 1), which we hope helps the reader to navigate in this rapidly expanding field of research. The review starts by description of the key pre-clinical and clinical trial data that has led to the approval of currently used exotoxin-targeted drugs.

DRUGGABLE STEP I - CELL SURFACE BINDING

Cell surface binding involving recognition of specific receptors is a necessary functional step for exotoxins. Many exotoxins such as superantigens and membrane-disrupting toxins also execute their effector functions at that particular cellular localization (Figure 1). A multitude of different drug modalities, including most of the currently FDA-approved and clinical trial drugs (Table 1, Figure 2), target this step of the functional pathway of exotoxins.

MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAbs) have several advantages in exotoxin-targeting such as high specificity, long in *vivo* half-life in circulation and good tolerability. In addition, mAbs do not merely act as passive extotoxin-neutralizing binders, but they also may execute beneficial Fc-fragment-mediated functions such as complement interactions and phagocytosis of exotoxin-mAb complexes. Antibody engineering technologies help in the design of enhanced versions, e.g. in affinity and immunogenicity also involving the possibility to combine two targeting specificities into a single product, i.e. the so-called bispecific antibodies. Tissue penetration is a drawback of these relatively large molecules (IgG, ~150 kDa). [30] The exotoxin-neutralizing mAbs are powerful prophylactic drugs, although vast amount of clinical and pre-clinical data also supports their post-diagnostic therapeutic use. As for now, all the clinically used exotoxin-targeted drugs are mAbs.

Raxibacumab (Abthrax®) and Obiltoxaximab (Anthim®)

Anthrax is a rare, but potentially lethal disease caused by rod-shaped, Gram-positive, spore-forming bacterium *Bacillus anthracis*. Inhalational anthrax drew global attention after the 2001 bioterrorist

attacks in the USA, which resulted in eleven confirmed cases of inhalational anthrax and five fatalities. The pathogenesis of inhalational anthrax is driven by the tripartite anthrax toxin complex [31]. The three different subunits - protective antigen (PA), lethal factor (LF), and edema factor (EF) - come together in binary combinations to form the lethal toxin (LT, PA+LF) and the edema toxin (ET, PA+EF). The PA recognizes specific receptors on the host cell surface, which leads to PA oligomerization, endocytosis of lethal toxin- and edema toxin-receptor complex, and release of LF and EF to the cytosol from the endosomal compartment [31]. The LF is a zinc metalloproteinase that inactivates mitogen-activated protein kinase-kinases (MAPKK), and EF is a calmodulin- and calcium-dependent adenylate cyclase that increases the level of intracellular cyclic adenosine monophosphate (cAMP) [31].

Obiltoxaximab is a chimeric PA-recognizing mAb, which has been engineered for higher affinity and for lower immunogenicity [32], building on the early work on mouse anthrax toxin-neutralizing antibodies [33] and mAb-PA interaction affinity enhancing mutations (1H variant) [34]. It is known at atomic resolution, in particular based on the work on its parental murine forms, that obiltoxaximab recognizes the receptor-binding region of PA [35] and thereby blocks PA-host cell receptor interactions. Raxibacumab is a fully human mAb binding to the PA, and acts in analogy to obiltoxaximab [36]. Obiltoxaximab was approved by FDA in March 2016. Raxibacumab got its FDA-approval in December 2012 and it was developed under the Project BioShield Act, which was launched by the US government in 2004. Obiltoxaximab has been shown to be well-tolerated among healthy volunteers in phase I trials and the most common adverse events included upper respiratory tract infections and hypersensitivity reactions [37]. The safety, tolerability and pharmacokinetics of raxibacumab in humans were evaluated with healthy volunteers in four sub-studies performed by Human Genome Sciences [36, 38]. These studies concluded that raxibacumab is safe, well-tolerated, and bioavailable after single intramuscular or intravenous dose [36, 38]. Most adverse events were mild to moderate in severity and did not significantly differ from placebo [36, 38]. Both drugs are indicated in adult and pediatric patients for the treatment of inhalational anthrax in combination with appropriate antibiotics and for prophylaxis of inhalational anthrax when alternative options are not available or are not appropriate. The recommended method of administration is intravenous infusion and the patients should be premedicated with oral or intravenous diphenhydramine to reduce the risk of infusion reactions [package inserts - Abthrax® (Raxibacumab), Rockville, MD, Human Genome Sciences, Inc, 2012; Anthim® (Obiltoxaximab), Pine Brook, NJ, Elusys Therapeutics, Inc, 2016].

The efficacy of Raxibacumab and Obiltoxaximab has been evaluated with animal experimentation utilizing rats, rabbits, dogs and macaques under the FDA Animal Rule. The main reason is that the nature of anthrax disease did not ethically justify human challenge studies. Initial therapeutic studies conducted in rats showed that raxibacumab increased survival when administered within 6 hours after a 24 hour toxin infusion [39]. Survival rate was lower in rats that received raxibacumab at 9 or 12 hours, and the survival rate also decreased with lower doses of raxibacumab [39]. Rats that received a prophylactic dosage of raxibacumab 24 hours prior to toxin infusion had a survival rate of 100% whereas all rats in the placebo group died [36]. In a study conducted with rabbits, animals receiving intravenous infusion of obiltoxaximab prior to exposure to anthrax spores had a survival rate of 100% whereas all saline-treated animals in control group died [32]. Rabbits that received obiltoxaximab 24 hours after the exposure had a survival rate of 80%, and when obiltoxaximab was given at 36h the survival rate decreased to 50% [32]. In the macaque mode, both raxibacumab and obiltoxaximab increased survival rates and the increase was dose-dependent [36, 40, 41].

Combinatorial therapeutic use with antibiotics, supportive care and anthrax toxin vaccination has also been studied by animal experimentation and clinical trials. The data in rabbits indicates that combining raxibacumab to levofloxacin improves survival compared to levofloxacin therapy alone [42]. Rabbit studies also support the use of obiltoxaximab-doxycycline combination [43]. In studies with canine model of anthrax toxin-associated shock it was shown that combination of hemodynamic support, i.e. titrated normal saline and norepinephrine infusions, and raxibacumab significantly improved survival compared to hemodynamic support alone [44]. Survival benefit of combination therapy was associated with increased diuresis, improved blood pressure and reduced demand on vasopressors and oxygenation [44]. The FDA-approved anthrax vaccine, anthrax vaccine absorbed (AVA), is mainly composed of adsorbed PA. A concern arose that in the case of postexposure prophylaxis with combination of AVA and raxibacumab, the AVA immunogenity could be decreased due to toxin-neutralizing activity of raxibacumab. However, in a recent open-label, randomized, multicenter study it was concluded that co-administrating raxibacumab with AVA does not significantly reduce immunogenicity of AVA [45]. There are currently two phase IV clinical trials with an objective to evaluate clinical benefit, safety and pharmacokinetics in patients treated with raxibacumab (NCT02177721) or obiltoxaximab (NCT03088111).

Bezlotoxumab (Zinplava®)

Clostridioides difficile infection (CDI) is the most common and costly cause of infectious diarrhea among hospitalized patients. It is caused by an anaerobic, Gram-positive, spore-forming, toxigenic bacterium and the disease usually follows antibiotic treatment due to dysbiosis of gut microbiota. The severity of the disease varies from asymptomatic carrier status to life-threatening pseudomembranous colitis [46]. *C. difficile*-induced colitis is commonly treated with enteral vancomycin, fidaxomicin and metronidazole, but after the primary treatment approximately 30% of patients have recurrent disease episode [47].

The major disease-causing virulence factors of *C. difficile* are the two large homologous clostridial exotoxins - toxin A (TcdA) and toxin B (TcdB). TcdA and TcdB are composed of four domains - glucosyl transferase domain (GTD), cysteine protease domain (CPD), pore-forming delivery domain and combined repetitive oligopeptide (CROP) domain. The CROP domain of TcdA/TcdB is involved in recognition of the host cell receptor triggering endocytosis. The acidic environment in the endosome leads to a conformational change in the pore-forming delivery domain which results in pore formation into the endosomal membrane and translocation of GTD and CPD domains to the cytosolic side of the endosome. Subsequently, cytosolic inositol hexakisphosphate (IP6) activates the CPD domain to cleave and release GTD into the cytosol. The GTD catalyzes UDP-glucose-consuming covalent glycosylation of cytosolic small GTPases such as Rac1, which results in actin depolymerization, cell rounding, and eventually cell death [46].

Bezlotoxumab is a TcdB-binding human mAb, which was identified via screening of hybridomas of TcdB-vaccinated HuMAb mice [48], i.e. mice that are transgenic for human and deficient for mouse immunoglobulin genes. Bezlotoxumab binds to the CROP domain, and prevents TcdB from binding to its receptor [49, 50]. Bezlotoxumab was developed by MassBiologics (MBL) in partnership with biopharmaceutical company Medarex. During the development of bezlotoxumab also an anti-TcdA human mAb (actoxumab) with similar mode of action as compared to bezlotoxumab was identified [48, 51], but it was later shown to lack efficiency in CDI [47]. Bezlotoxumab (Zinplava®) was FDA-approved in 2016 for the use in clinical practice to reduce recurrence of CDI in adult patients (18 years or older) who are treated with standard of care antibiotics for *C. difficile* infection and are at high risk for CDI. Bezlotoxumab is administered via intravenous infusion [package insert - Zinplava® (Bezlotoxumab), Whitehouse Station, NJ, Merck & CO, Inc, 2016].

In preclinical cell culture-based studies bezlotoxumab, and also actoxumab, were shown to neutralize toxin activities of several *C. difficile* strains, including the epidemic ribotypes BI/NAP1/027 and BK/NAP7/078 [52]. In multiple murine models of CDI, intraperitoneally administered actoxumab-bezlotoxumab mixture reduced the tissue damage and inflammatory response in the gut wall [53]. The pharmacokinetics and safety of bezlotoxumab was evaluated in two large multicenter trials [47]. The safety profile of bezlotoxumab was similar to that of placebo and no antibodies against bezlotoxumab was detected after treatment [47]. In phase II study the combination of actoxumab and bezlotoxumab lowered the risk of recurrent CDI among patients that also received standard-of-care when compared to placebo [54]. Phase III trials for actoxumab and bezlotoxumab included two international, multicenter, double-blind, randomized and placebo-controlled studies (MODIFY I & MODIFY II) in which the effect of actoxumab and bezlotoxumab were studied on patients with primary or recurrent *C. difficile* infection [47]. Primary endpoint in these studies was recurrent infection, i.e. new episode after initial clinical cure, within 12 weeks after infusion. In both trials the risk of recurrent CDI was significantly lower in the bezlotoxumab group than in the placebo group (MODIFY I: 17% vs. 28%, MODIFY II: 16% vs. 26%). Subgroup analyses revealed that in the subpopulations at high risk for recurrent infection (Age >65, history of CDI, compromised immunity, severe CDI) or for an adverse outcome, both groups that received bezlotoxumab had lower rate of recurrent infection than in the placebo group. Among high-risk patients, who were hospitalized at the time of infusion, bezlotoxumab decreased the rate of hospital readmission within 30 days. However, bezlotoxumab or actoxumab did not increase the probability on initial clinical cure. It was also shown that the patients that had no risk factors for recurrent CDI did not benefit from additional treatment with bezlotoxumab. Recently, more analysis of the MODIFY I-II data has been published, e.g. [55] [56], that together with the real-world efficacy analysis in clinical practice, such as in Finland [57], support the use of bezlotoxumab in CDI. In conclusion, bezlotoxumab has been shown to be safe and effective way to reduce the risk of recurrent *C. difficile* infection among the high-risk patients. Even though the cost of bezlotoxumab treatment is not negligible, cost-effectiveness analyses has shown to favor treatment with bezlotoxumab [58]. According to ClinicalTrial.gov, there are five phase IV (NCT04626947, NCT03880539, NCT03937999, NCT03756454, NCT04415918) one phase III (NCT03182907), one phase II (NCT03829475) and two case-control studies (NCT04317963, NCT04075422) ongoing with connection to bezlotoxumab. All trials are currently in a recruiting phase.

ASN100

Staphylococcus aureus is a Gram-positive common bacterial commensal of humans. It is also a major opportunistic pathogen, and the global disease burden of *S. aureus* infections is remarkable. The severity of *S. aureus* infections ranges from mild skin infections, e.g. abscesses and impetigo, to severe and potentially life-threatening infections as pneumonia, septicaemia, osteomyelitis and endocarditis. Despite the appropriate antibiotic treatment, the mortality in severe infections remain high. The appearance of methicillin- and vancomycin-resistant *S. aureus* strains is concerning as infections are becoming more demanding to treat [59]. *S. aureus* produces tens of different exotoxins, which can be divided into three major groups - exfoliative toxins, superantigens and membrane-disrupting toxins [7]. Membrane-disrupting toxins can be further divided into four groups - α-toxin, hemolysin-β, leukocidins and phenol-soluble modulins (PSMs). Presumably the most renowned *S. aureus* toxin is the pore-forming α-toxin also known as Hla or α-hemolysin. It is a water-soluble polypeptide, secreted as a monomer by majority of clinical *S. aureus* strains [7]. After binding to a receptor on the target cell surface, it quickly oligomerizes and forms a transmembrane β-barrel pore leading to profound cell signaling effects and eventually to cell lysis [7]. There are currently five known leukocidins in *S. aureus* strains associated with human infections - panton-valentine leukocidin (PVL or LukSF-PV), two γ-hemolysins (HlgAB, HlgCB), LukED and LukAB/HG [7]. Leukocidins are are composed of two protein subunits designated as S- and F-subunits [7]. The S-subunits bind to the host cell surface receptor leading to recruitment and dimerization of the F-subunits [7]. The LukGH also appears to dimerize in solution [60]. Oligomerization of the F-subunit dimers eventually results in the leukocidin pore formation [7].

ASN100 was developed by Arsanis Biosciences GmbH, based on screening of high diversity yeast surface displayed human IgG1 libraries [60, 61]. ASN100 is composed of two fully human IgG1 mAbs, ASN-1 [61] and ASN-2 [60]. ASN-1 neutralizes a-toxin and the leukocidins LukSF-PV, LukED, HlgAB and HlgCB via a common conformational epitope shared between a-toxin and leukocidin F-subunits [61]. Apparent mode of action is masking the phosphocholine-binding pockets of a-toxin and leukocidin F-subunits and thereby prevention of membrane interactions required for pore maturation [61]. ASN-2 neutralizes the fifth leukocidin, LukAB/GH [60]. Interestingly, ASN-2 recognizes the S- and F-subunit dimeric structure, yet leading to the same mode of action than ASN-1 preventing leukocidin interactions with the target cells. During the first preclinical *in vitro* studies, ASN-1 was shown to inhibit a-toxin-mediated lysis of epithelial cells and leucocidin-mediated destruction of phagocytes and human erythrocytes [61]. The ASN-2 protected polymorphonuclear phagocytes from LukGH-

mediated lysis [60]. Both ASN-1 and ASN-2 were needed to protect human leukocytes from cytotoxicity after exposure of culture supernatants of different *S. aureus* strains [62]. ASN100, but also ASN-1 alone, was able to protect the morphology of 3D human tracheal/bronchial mucociliary epithelial tissue culture infected with *S. aureus* [62]. In murine models, passive immunization with ASN-1 before intranasal or intravenous challenge with *S. aureus*, prevented lethal pneumonia and sepsis [61]. Also a therapeutic effect was observed, when ASN-1 was administered 2 hours after intranasal challenge in combination with linezolid [61]. In another study, the prophylactic efficacy of ASN100 in rabbit *S. aureus* pneumonia model was evaluated [63]. In this study, ASN100 was shown to increase survival in dose-dependent manner when given intravenously prior to intratracheal exposure of *S. aureus* [63]. Also a reduced macroscopic and microscopic lung pathology and bacterial burden were observed [63]. Pharmacokinetic analysis of bronchoalveolar lavage (BAL) fluid showed ASN100 penetration to lung epithelial lining fluid at 24 hours after administration with peak levels of ASN100 appearing at 48 hours [63].

The safety, tolerability and pharmacokinetics of ASN100 was evaluated in randomized, double-blind, phase I study with healthy volunteers [64]. The subjects were randomized to receive ASN-1, ASN-2, ASN100 or placebo with different dosages. To assess the pharmacokinetics of ASN100 in lung epithelial lining fluid, BAL fluid samples were collected from twelve subjects. Study revealed that ASN100 and its individual components were safe and tolerable at doses up to 8000mg. No dose-limiting toxicities were observed during the study. The most reported adverse effects were headache, nasopharyngitis and symptoms of gastroenteritis, but also single events of somnolence, vertigo and dizziness were observed. All adverse events were mild or moderate in severity and resolved without medical interventions. Higher dosages did not increase the risk of adverse events. ASN-1 and ASN-2 seemed to have linear pharmacokinetics with a half-life of 20 to 36 days after intravenous administration. Both components were detectable in BAL fluid already at 24h or 48h and remained detectable at least out to day 30. Also, the toxin neutralization activity of ASN-1 and ASN-2 was preserved in human sera. Anti-ASN100 antibodies were not detected in significant quantities [64]. The effect of ASN100 for prevention of *S. aureus* pneumonia in mechanically ventilated patients was studied in multicenter, double-blind, single-dose, placebo-controlled trial (NCT02940626, study duration 2016 - 2018). In this study, participants (n = 155) were selected by culturing an endotracheal aspirate to identify those who are heavily colonized with *S. aureus.* Subjects were randomized to receive either ASN100 or placebo. The primary endpoint was to determine the proportion of patients which had or had not developed *S. aureus* pneumonia after single intravenous dose of ASN100. After

preplanned interim analysis of 118 subjects, the data review committee informed that the study was unlikely to meet its primary endpoint with statistically significant difference and the trial was terminated. However, patients were followed for adverse effects after the trial termination. The results of the phase III trial have not been published, nor it is known how AS100 development pipeline is being continued. The mode of action of both ASN-1 and ASN-2 is blockage of the pore oligomeric assembly, which might be more problematic and less efficient as compared to receptor binding blockage.

There are currently also other *S. aureus* exotoxin-targeted mAbs in clinical trials (Table 1). MEDI4893 (suvratoxumab) is a human mAb that binds to *S. aureus* a-toxin, in particular to an area sterically preventing host cell surface receptor binding and thereby subsequent a-toxin oligomerization [65]. In mouse model of *S. aureus* pneumonia, for instance, passive immunization with MEDI4893 was shown to decrease mortality and bacterial burden in the lungs [66]. In phase I trial, MEDI4893 was well tolerated among subjects and no serious adverse effects were reported [67]. The phase II trial of MEDI4893 (NCT02296320, study duration 2014 - 2018) has been conducted. No publications of this study have been released. AR-301, also known as Salvecin, is another mAb that binds and neutralizes a-toxin. No preclinical data has been published, but it is known that AR-301 was discovered by screening B cell repertoire of *S. aureus* pneumonia patient for mAbs with a-toxin neutralizing activity [68]. Treatment of *S. aureus*-challenged mice with AR-301 either prophylactically or therapeutically, was effective [68]. In a phase I/II trial, the safety and efficacy of AR-301 was evaluated with intensive care unit patients with severe microbiologically confirmed *S. aureus* pneumonia. The results showed that AR-301 was well-tolerated and no serious adverse effects were reported. In a subgroup analysis of patients with ventilator-associated bacterial pneumonia, the ventilation duration was shorter among patients who received AR-301 as compared to placebo [68]. The phase III trial of AR-301 is currently in a recruiting phase (NCT03816956).

Shigamabs

Some strains of *Escherichia coli*, such as Shiga toxin-producing *E. coli* (STEC), can cause a severe foodborne disease. Clinical manifestations of STEC infections vary from asymptomatic carriage to severe hemorrhagic colitis. The most severe complication of STEC infection is hemolytic uremic syndrome (HUS), which is a thrombotic disorder, characterized by microvascular thrombi, microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Significant portion of

patients suffering from HUS need renal dialysis and particularly children and elderly are more susceptible to complications and death [69]. Administration of antibiotics in these STEC infections has long been controversially associated with increased risk of hemolytic uremic syndrome (HUS). In a recent review article [70], it was concluded that the risk of HUS seems to associate to the particular STEC strain causing the infection and to the antibiotic class used in the treatment. Because of the potential negative effect of antibiotics, other alternative therapeutic agents against STEC have been under development and the first Shiga toxin-neutralizing mAbs were introduced in the 1980s, e.g. [71].

E. coli Shiga toxins (Stx1 and Stx2), and the canonical *Shigella dysenteriae* Shiga toxin (Stx), are AB5 topology exotoxins with extremely potent cytotoxicity [72]. The pentameric B-subunit mediates Shiga toxin binding to the host cell surface receptor globotriaosylceramide (Gb3), mainly displayed on the surface of endothelial cells. The receptor docking is followed by endocytosis, and retrograde trafficking to the Golgi and ER with subsequent release of the catalytic A-subunit from the ER into the cytosol. The A-subunit has N-glycosidase enzymatic activity, i.e. it recognizes 28s rRNA of the 60S ribosome subunit and depurinates one specific adenine residue. This relatively small modification leads into blockage of protein synthesis and subsequent cell death [72].

Shigamabs is a combination of two chimeric mAbs cαStx1 and cαStx2, which recognize and neutralize Stx1 and Stx2 [73]. The development pipeline is based on mouse mAbs, naimly Stx1 B-subunit recognizing 13C4 [71] and Stx2 A-subunit recognizing 11E10 [74]. The 13C4 mAb neutralizes Stx1 via blockage of Stx1-host cell receptor interaction [75], whereas 11E10 appears to alter the sub-cellular trafficking of Stx2 [76]. Thorough efficacy studies of Shigamabs in mice have been published [73]. CD-1 mice were used to evaluate the efficacy of cαStx1. The efficacy of cαStx2 was evaluated in both CD-1 mice and in streptomycin-treated, orally infected mouse model of STEC infection. During the study, mice were either orally infected with a lethal dose of Stx2-producing STEC strain B2F1 or they were given intraperitoneal injection of purified Stx1 and/or Stx2 (LD_{50}). The cαStx1, cαStx2 or combination of both (Shigamabs) were given intravenously either before or after infection or toxin administration. The results showed that cαStx1 protected CD-1 mice when given either before or after toxin injection. With Stx2-injected mice treated with cαStx2, the results were similar. In mice infected with B2F1, 0.1mg/kg dose of cαStx2 protected the mice when given at 24 or 48 hours after the infection. The cαStx2 was also proven to be effective when administered intramuscularly. In CD-1 mice that were injected simultaneously with Stx1 and Stx2, both cαStx1 and cαStx2 were required to

protect the mice. Mice that received a combination of cαStx1 and cαStx2 1 hour prior to intoxication had a survival rate of 70% [73].

The tolerability and pharmacokinetics of cαStx2 have been evaluated in a phase I trial [77]. In this open-label, nonrandomized study, 17 healthy volunteers were divided in four groups to receive escalating doses (0.1-10mg/kg) of cαStx2 by intravenous infusion. Among the subjects, most common adverse effect was headache, which was reported by 52% (n=9). 35% (n=6) of the subjects did not report any symptoms at all during the surveillance. Two out of three volunteers who received highest dosage of cαStx2 (10mg/kg) reported mild joint pain, which resolved spontaneously. All three of them also reported mild headache. Mild and spontaneously resolving elevations in serum liver enzyme levels (ALT and/or AST) were also detected and one volunteer had leukopenia of 2 300 cells/µl on day 3. However, it is not certain that these mild abnormalities in laboratory values were related to the infused mAb. Anti-chimeric antibodies were detected in 24% of the volunteers on day 56, but the presence of these antibodies did not seem to have an effect on the clearance of cαStx2. The tolerability and pharmacokinetics of cαStx1 was evaluated in two single-center, open-label, nonrandomized, dose-escalation phase I studies [78]. Also, the safety of combined infusion of cαStx1 and cαStx2 was evaluated. Subjects (n=26) were healthy adult volunteers, who received an intravenous infusion of cαStx1, cαStx2 or both at dose of 1mg/kg or 3mg/kg. 69% (n=18) of volunteers reported at least one adverse effect, but no severe adverse effects were reported among the subjects. The most common adverse effects were headache and mild somnolence, symptoms of upper respiratory tract infections and gastrointestinal inconveniences. Data analysis showed that there were no association between the probability of adverse effects and the cαStx dosage. Administration of cαStx1/cαStx2 combination did not either increase the risk of adverse effects. The pharmacokinetic profile of both cαStx1 and cαStx2 were similar and simultaneous infusion of both antibodies did not have effect on pharmacokinetics. Anti-chimeric antibodies were only detected on day 57 in one volunteer, who had received cαStx2.

The safety, tolerability and efficacy of Shigamab was evaluated in a randomized, placebo-controlled, multicenter Phase II trial called SHIGATEC (NCT01252199). The subjects (n=45) were children aged between 6 months to 18 years, diagnosed with Shiga toxin-producing bacterial infection and bloody diarrhea. The results have not been released, but the drug was mentioned in one review article to be well tolerated and safe according to the preliminary data [79]. However, the efficacy of Shigamabs

in preventing HUS is still unknown. Shigamabs was developed by Thallion Pharmaceuticals Inc. in collaboration with LFB Biotechnologies. In 2013 it was announced that the collaboration between Thallion and LFB ended and all the rights of the Shigamabs program reverted to Thallion. However, in 2017 Sun Pharmaceutical Industries Ltd. acquired Thallion and the transaction is believed to assist the development of Shigamabs. At the time of the acquisition, Sun Pharma estimated that the commercialization of Shigamabs would take around seven to eight years. There is a possibility that the financial circumstances between Thallion, LFB and Sun Pharma might have an impact on the developmental pipeline of Shigamabs as well as data release of the clinical trials.

Several other mAbs against Shiga toxins have also been developed. Most notably, the Stx2-binding TMA-15, also known as urtoxazumab, proceeded to phase I trial and was shown to be safe and well tolerated in humans [80]. This developmental pipeline is based on humanized mouse mAb, VTm1.1 [81, 82], which binds to the pentameric B-subunit of Stx2. In preclinical studies, treatment with TMA-15 up to 24 h after infection ameliorated the lethal Stx2-producing STEC strain B2F1 challenge in mice [83]. However, the urtoxazumab dosage needed to protect the STEC-infected mice appears to be significantly higher as compared to cαStx2 [73]. The efficacy of urtoxazumab has also been evaluated in a gnotobiotic piglet model, and the results suggest that urtoxazumab might potentially reduce post-EHEC neurological sequelae [84]. The developmental future of urtoxazumab remains unclear.

hu1B7/hu11E6 cocktail

In addition to the FDA-approved and the clinical trial mAbs (Table 1), there are a number of exotoxin-targeted mAbs in pre-clinical development (Table 2, Suppl. Table 1). Many of these are in an early state. A notable difference is the developmental pipeline focused on pertussis toxin, which is the major virulence factor of *B. pertussis* [8]. The Gram-negative bacterium *B. pertussis* is the etiological agent of the whooping cough, i.e. pertussis. Whooping cough is a globally distributed acute respiratory disease, affecting all age groups [85]. However, infants and young children comprise the highest risk cohort, where the disease may lead to death despite hospital intensive care and use of antibiotics [85]. Despite the global vaccine campaign pertussis remains endemic, causing outbreaks in many regions of the world, and the disease incidence is increasing [86]. Moreover, macrolide resistant *B. pertussis* strains have been reported [87, 88]. Especially young children who still lack the vaccine-induced protection against whooping cough could benefit from pertussis toxin-neutralizing mAbs. The young whooping cough patients, in contrast to adults, are typically diagnosed very early and

thereby could possess a therapeutic window to interfere with the pertussis toxin-induced pathology. Exposed family members of the whooping cough patients could be an additional patient group subjected to a prophylactic administration of pertussis toxin mAbs, possibly in combination with antibiotics.

Humanized pertussis toxin-neutralizing monoclonal antibodies hu1B7 and hu11E6 have been developed [89-91], and even combined into a single bispecific mAb [92], building on the early mouse anti-pertussis toxin antibody studies, e.g. [93]. Both hu1B7 and hu11E6 antibodies, either individually or as a cocktail, form multivalent complexes with soluble pertussis toxin that bind the FcγRIIb receptor more tightly than antibodies alone [90]. This indicates that the antibodies could accelerate pertussis toxin clearance via immune complex formation. However, hu11E6, and to some extent hu1B7, also prevents pertussis toxin binding to its cell surface receptor. In addition, hu1B7 appears to trap pertussis toxin at or near the cell surface by either interfering with endocytosis or with the early steps in retrograde trafficking of pertussis toxin [90]. It is very encouraging that a hu1B7/hu11E6 cocktail has a prophylactic and therapeutic effect in mouse (intraperitoneal route) and adult baboon (intravenous route) pertussis models, respectively [91]. Moreover, the most recent experimentation with hu1B7 monotherapy (intravenous route) in an infant baboon pertussis model demonstrates a potent prophylactic effect [89].

ANTIBODY FRAGMENTS

Antibody fragments include the mono- and bivalent antigen-binding fragments (Fab) and $F(ab')_2$, respectively, single-chain variable fragments (scFvs) and single domain antibodies, i.e. VHH nanobodies derived from the heavy-chain-only camelid immunoglobulins [94] (Figure 2). Antibody fragments can offer several advantages over the use of conventional mAbs. For example, they can be produced more easily, generally using microbial expression systems, which results in faster cultivation, higher yields, and lower production costs. Their small size also allows better tissue penetration and they may have reduced immunogenicity. Antibody engineering also utilizes antibody fragments, e.g. linking the most efficient fragments with Fc-region to engage effector functions of entire mAbs. Major drawback is a short serum half-life, which, however, can be engineered. The exotoxin-neutralizing antibody fragments are powerful prophylactic drug leads, although vast amount of pre-clinical data also supports their post-diagnostic therapeutic use.

In recent years there has been substantial amount of work done towards developing antibody fragments that inhibit the action of bacterial exotoxins (Suppl. Table 1). These fragments include VHHs, Fab and $F(ab')_2$, scFvs and a variety of fusions of them. VHH, Fab and scFv fragments are often used in phage display selections and displays, and for initial characterization, but eventually engineered to IgG, IgG-like fusion scFv-Fcs or VHH-Fcs, as exemplified by the work done on staphylococcal superantigenic exotoxin B [95] and clostridial TcdB [96] and BoNT/A [97]. In their recent work Lam *et al*. [98] investigated high resolution structures and neutralizing mechanisms of unique VHHs against BoNT/A1 and BoNT/B1 of *C. botulinum*. BoNT molecule is composed of a light chain (LC, the protease domain) and a heavy chain (HC), which is comprised of an N-terminal translocation domain and a C-terminal receptor-binding domain. The receptor-binding domain determines neuronal specificity by recognizing a polysialoganglioside, e.g. GT1b, and a protein receptor, i.e. synaptotagmin, Syt, for Bont/B and glycosylated synaptic vesicle protein 2, SV2, for BonT/A. BoNT/B has an additional hydrophobic loop in the receptor-binding domain, which interacts with host membrane lipids. The BoNT/B targeting VHHs were found to bind to the C-terminal subdomain of BoNT/B, in particular in such a way that the BoNT/B-polysialoganglioside/Syt/lipid receptor interactions are pevented. In contrast, BoNT/A targeting VHHs blocked either the membrane insertion of the translocation domain or interfered with the unfolding of the protease domain. By connecting two VHHs with proximal epitopes and complementary neutralizing mechanism with flexible spacer, bifunctional VHH heterodimers (VHH-based neutralizing agents, VNAs) were created. These VNAs with dual epitope binding mode showed superior potency in mouse BoNT/A or BoNT/B co-intoxication assay (toxins and CHHs mixed prior to intraperitoneal injection) as compared to similar VHHs that are unable to bind two epitopes simultaneously. Moreover, the VNAs also protected mice against BoNT/A1 and BoNT/B1 when administered 30 or 60 min prior to toxins. The described VHH targeted epitopes are identical or moderately conserved between different BoNT/A and BoNT/B subtypes, respectively, so these VHHs likely have some affinity toward most or all BoNT/A and BoNT/B subtypes.

ANTIBODY MIMETICS

Antibody mimetics represent an alternative class of therapeutics able to overcome some of the limitations of mAbs, while still possessing many of their benefits, e.g. high target binding affinity and specificity [99]. Antibody mimetics is a heterogeneous group including protein domains such as designed ankyrin repeat proteins (DARPins) and centyrins. Antibody mimetics are small (<20 kDa) single-domain scaffolds that are thermostable, highly engineerable and can be produced in

microorganisms or even be completely synthesized chemically. As many of these scaffolds are derived from human proteins, they possess low immunogenicity. Owing to their small size, enhanced tissue penetration is also expected. Their serum half-life is short. However, this can be extended by engineering, e.g. with PEGylation or conjugation with serum albumin [100].

Two papers have recently been published on the use of DARPins to neutralize exotoxins [101, 102]. DARPins are derived from natural ankyrin repeat proteins, which are among the most abundant binding proteins found in the human genome [103]. DARPins are small, single domain proteins (~15 kDa), consisting of three repeat modules - N-terminal capping repeat (N-cap), varying number of internal ankyrin repeats, and a C-terminal capping repeat (C-cap) (Figure 2). A series of monomeric and dimeric DARPins with potent neutralization activity for *C. difficile* TcdB have been developed. These DARPins neutralized TcdB from the laboratory strain VPI 10463 (ribotype 087), the clinical strain M68 (NAP9/CF/017) and the hypervirulent strain (NAP1/BI/027). The monomeric DARPins against TcdB interfered with the interaction between TcdB and its receptors chondroitin sulfate proteoglycan 4 (CSPG4) and Frizzled receptor 2 (FZD2), respectively, by binding to the delivery domain of TcdB. Dimers were generated by combining the monomers, which interfered with the CSPG4 and FZD2 receptor docking.*In vitro* studies showed that the best DARPins, the dimeric DLD-4 against the TcdB from strains VPI 10463 and M68, and monomeric D16 against the hypervirulent strain (ribotype 027) TcdB had superior TcdB-neutralization potencies as compared to the FDA-approved mAb bezlotoxumab. *In vivo* efficacy of the dimeric DLD-4 has also been studied in two mouse models, intraperitoneal injection and cecum injection models, against TcdB challenge. Significant increase in the mouse survival with intraperitoneal injection of pre-incubated mix of TcdB and DLD-4 was monitored, indicating that DLD-4 possesses significant toxin-neutralization ability *in vivo*. However, only a minor survival advantage was observed with the cecum injection model in mice receiving combination of TcdB and DLD-4 compared to TcdB alone. This was due to the unexpectedly poor resistance of DLD-4 against the protease activity of gut trypsin and chymotrypsin. This shortcoming might be overcome by obtaining protease-stable variants of this DARPin with second-generation engineering. It remains unclear whether the DARPins would attenuate TcdB-induced symptoms after a systemic TcdB exposure.

Centyrins that neutralize the bicomponent leukocidins PVL, HlgAB, HlgCB, LukED, and LukAB of *S. aureus* have been identified [104]. Centyrins are small (~10 kDa) globular proteins

derived from a consensus sequence of the 15 fibronectin type III (FN3)-binding domains of the human tenascin-C protein [105, 106]. These centyrins blocked binding of bicomponent leukocidins to their host cell surface receptors. Some of the centyrins exhibited cross-reactive properties targeting leukocidins that share strong amino acid sequence conservation between their subunits, e.g. HlgCB and LukSF-PV. Centyrins also protected human phagocytes from toxin-mediated killing. In murine models of LukED or HlgAB intoxication, centyrins and centyrin-serum albumin fusion constructs premixed with toxins before intravenous administration or centyrins given prophylactically before toxin administration were shown to protect the mice. Centyrin-serum albumin fusion constructs also markedly improved survival and reduction of bacterial burdens when given 4 hours after intravenous infection with highly virulent MRSA. With further engineering, these biologic agents with toxin neutralizing activity could have a potential clinical utility in the treatment and prevention of serious staphylococcal infections.

RECEPTOR ANALOGS AND NEUTRALIZING SCAFFOLDS

Receptor analogs and neutralizing scaffolds is a heterogeneous group of exotoxin-targeted drug leads. They prevent the interaction of exotoxins with their host cell receptor structures, i.e. lipids, carbohydrates or proteins, via molecular mimicry or they reduce the bio-availability of the soluble forms of exotoxins via sequestration. Obvious benefits include generally good tolerability and long half-lifes as many of these are based on natural host cell surface structures. These modalities include some of the most early attempts to develop exotoxin-neutralizing strategies. However, recent interesting developments have emerged, e.g. combinations of multiple modes-of-action into a single product. As for now, three development pipelines have entered clinical trials, SYNSORB-Pk, Tolevamer and CAL-02 (Table 1).

SYNSORB-Pk is a polymer with the Shiga toxin host cell surface receptor Gb3 trisaccharide moiety covalently linked to silicon dioxide particles via a defined linker [107]. Orally administrated SYNSORB-Pk was safely tolerated by healthy adult volunteers in a phase I study without any evidence of toxicity [107]. In the same study, SYNSORB-Pk remained active upon passage through the gastrointestinal tract, i.e. it neutralized Shiga toxin in ETEC-positive stool samples from patients with hemolytic uremic syndrome (HUS) or hemorrhagic colitis [107]. However, a multicenter double-blind phase III clinical trial demonstrated that SYNSORB-Pk was ineffective at reducing the severity of diarrhea-associated HUS in pediatric patients [108]. There are a number of possibilities to explain the

negative outcome, one being simply the lack of efficiency. However, only third of the enrolled diarrhea-associated HUS patients had viable STEC or free Shiga toxins in their stool samples [108]. The authors proposed that the SYNSORB-Pk intervention might have started too late to have a therapeutic effect, i.e. Shiga toxin had already entered the circulation. The SYNSORB-Pk development pipeline has been on apparent hold since the discouraging phase III clinical trial was conducted 20 years ago.

Tolevamer, formerly known as GT160-246 and GT267-004, is a high molecular weight (≥400 kDa), soluble linear polymer of styrene sulfonate that binds and neutralizes *C. difficile* toxins TcdA and TcdB *in vitro* and *in vivo* [109-111]. The exact binding mode is not known. The GT160-246 version was found to be non-inferior, i.e. not worse, to vancomycin in mild to moderate CDI in a phase II clinical trial [112]. The GT160-246 version was well tolerated in this promising phase II trial, but a common side effect was hypokalemia [112]. Therefore, a new oral solution formulation with a mixed potassium sodium salt of Tolevamer (GT267-004) was developed [113]. The GT267-004 version demonstrated lower hypokalemia side-effects and was well-tolerated in a phase I trial [113]. However, the GT267-004 version was found to be inferior, i.e. worse, to standard antibiotic therapy for CDI conducted either with vancomycin or metronidazole in two multinational phase III trials [114]. This discouraging result could, in part, be explained by the fact that Tolevamer interacts less tightly with TcdB as compared to TcdA *in vitro* [110]. Phase III clinical trials with a different drug modality, intravenously-administrated mAbs, demonstrated efficacy only with TcdB neutralization [47] (see more above). Animal experimentation and prevalence of TcdA- and TcdB-encoding genes in clinical *C. difficile* isolates also indicates dominance of TcdB in disease pathology [46]. Tolevamer development pipeline has been on apparent hold since the discouraging phase III clinical trials were conducted 15 years ago.

The exotoxin-targeted drug development pipeline contains a plethora of other approaches to neutralize exotoxins with receptor analogs and neutralizing scaffolds (Suppl. Table 1), as exemplified by the work on Shiga toxins. The Daisy [115], Starfish [116] and Super Twig [117, 118] concepts are polyvalent Shiga toxin carbohydrate receptor analogs, which have been efficient in pre-clinical *in vitro* and *in vivo* experimentation. However, clinical trials have not been conducted on these early drug candidates. An interesting variant concept of receptor analogs, which also acts as an efficient neutralizing scaffold, relies on the use of a recombinant bacterium that expresses a mimic of the Shiga toxin receptor globotriaosyl ceramide (Gb3) on its surface [119]. This engineered bacterium was also

effective *in vivo*, protecting mice from otherwise fatal STEC infection [119]. This concept was recently upgraded via the development of Gb3 receptor mimic bacterial ghosts (BGs) [120]. BGs are empty, non-living bacterial envelopes of Gram-negative bacteria that are not classified as genetically modified organisms, and thereby could remove barriers in the development of bacterium-displayed Gb3 receptors towards clinical use [120]. Recently, nanoparticles functionalized with lipids, receptors, receptor fragment or peptides have been developed as one type of neutralizing scaffolds. For example, calcium phosphate nanoparticles loaded with peptides derived from the CTLD4 domain of the human mannose receptor, MRC-1, that interacts with the conserved cholesterol-binding loop of cholesterol dependent cytolysin (CDCs) [121, 122] were shown to be able to improve survival and bacterial clearance in *in vivo* models of pneumococcal infection [121].

Many toxins, especially many cytolysin (cholesterol dependent cytolysins, CDCs), bind preferentially to cholesterol-containing membranes. By using membrane mimicking neutralizing scaffolds, such as nanoparticles coated with lipids, liposomes containing cholesterol at higher than physiological levels [123], exosomes [124] or so called biomimetic nanosponges composed of a red blood cell membrane (RBCM) fused to a polymer nanoparticle core, it is possible to inhibit wide variety of exotoxins from binding to the host cell membrane [125-128]. Nanosponges have the same repertoire of cell membrane receptors as their host cell, so they can act as non-specific toxin decoy strategy with a broad ability to sequester and neutralize various bacterial exotoxins. One application of these nanosponges is to include an antibiotic [127, 128] or other bacterium-targeting molecule [129] into the nanoparticle core. When the exotoxins bind and destroy the RBCM coating, the antibacterial compound trapped inside the nanoparticle is released. These approaches aim for more targeted delivery of the antibiotic by releasing it in the site of infection. Whole red blood cells can also be used as scaffolds to prolong the circulatory half-life of exotoxin-neutralizing molecules. Genetically engineered red blood cells expressing chimeric proteins of camelid VHHs with blood group antigens Glycophorin A or Kell were shown to confer long-term protection against botulinum neurotoxin A when transfused to mice exposed to highly lethal doses of BoNT/A [130]. These recent developments exemplify the concept where different drug modalities are being combined to a single therapeutic, to increase efficiency and targeting capability. One of the exciting new approaches relies on the use of liposomes to treat bacterial infections. CAL-02 consists of a mixture of liposomes that create artificially large and stable liquid-ordered lipid microdomains and function as docking sites for a large range of bacterial toxins [131]. CAL-02 recently entered phase I trial in severe pneumococcal pneumonia , and it was shown to possess a promising safety profile and tolerability when administered by infusion [131].

DOMINANT NEGATIVE MUTANTS

Several exotoxins, in particular membrane-disrupting toxins such as a-toxin of *S. aureus* [132] require assembly and oligomerization at the host cell surface in order to execute their cytotoxic effector activities. While deciphering the mechanisms by which leukocidin LukED, another pore-forming exotoxin of *S. aureus*, targets and kills host cells, short glycine-rich motifs within the stem domains of LukE and LukeD were identified as necessary structural elements [133]. Remarkably, mutant leukocidin subunits lacking these motifs behaved as dominant-negative toxins and neutralized the cytolytic activity of wild-type leukocidins *in vitro* in cell cultures [133]. The mutant leukocidin subunits appeared to bind on the host cell surface receptors, and also were able to interact with the wild-type leucocidin subunits [133]. The data implies that mechanistically the dominant negative mutant subunits and wild-type subunits of leukocidins hetero-oligomerize but assemble into a defective pore complex, thereby inhibiting toxicity. It is interesting that intravenous administration of dominant negative mutants had a prophylactic and therapeutic effect in mouse models of intravenous leukocidin challenge and *S. aureus* infection, respectively [133].

The above study on *S. aureus* leukocidins is preceded by other similar studies proposing the use of dominant negative mutants to prevent the functions of membrane-disrupting toxins, e.g. on *Clostridium perfringens* e-toxin [134], *Helicobacter pylori* VacA [135] and *Bacillus anthracis* anthrax-toxin [136-138]. These examples imply that the use of dominant-negative mutants is a feasible strategy to neutralize multimeric membrane-disrupting toxins. However, efficient and broad development of this drug modality would require an in-depth high-resolution structural knowledge, allowing rational mutant design, that is not currently available for many membrane-disrupting toxins. Also, the number of mutations that inactivate the toxins is expected to be substantially greater than the number of mutations that lead to a dominant-negative phenotype. In the end, this means more screening work and slower progress. One additional potential problem, based on the recent *S. aureus* leukocidin work [133], appears to be the short half-lifes of the dominant-negative mutants. Intravenously administrated dominant negative mutants were protective if they were given no more than 5 hours before the wild-type leukocidin challenge [133]. Parallel experiments done with *S. aureus* infections also indicated problems with the half-lifes [133]. As for now, it appears that the dominant negative mutants of exotoxins remain as very useful basic research tools, rather than efficient emplates for drug development. However, one variant of the dominant negative approach is the use of exotoxin-derived

peptides, which destabilize the exotoxin structure and thereby inhibit the cellular toxicity, as exemplified with TcdB of *C. difficile* [139]. These kinds of peptides are expected to have better pharmacokinetic properties as compared to full length protein subunits.

SMALL MOLECULES

Small molecules have been the traditional basis for drug development and almost two-thirds of approved medicines are either naturally derived or synthetic small molecules [140]. Small molecule drugs typically have no more than 100 atoms, and they are no bigger than 1000 g/mol or 1 kDa in size. Small molecules have distinct advantages as therapeutics. Due to their small size, small molecules penetrate tissues efficiently, and may also enter the cell allowing effective targeting of cytosolic processes. Most can be formulated and optimized for oral administration, allowing absorption into the bloodstream and thereby access to the whole body. Due to the possibility to produce small molecules via chemical synthesis, the production costs are typically lower as compared to other modalities, e.g. mAbs. Small molecules can be designed to engage biological targets, mostly proteins, by various modes of action with high resolution structure-based rational drug design approaches. These include binding to and inhibition of enzyme active sites, binding to allosteric sites influencing enzyme activities and structural transitions, and binding to regions of proteins mediating interactions with other proteins, i.e. protein-protein interaction (PPI) inhibitors. In addition, high-throughput screening with small molecule compound or fragment libraries using cell-based or *in vitro* biochemical assays allows efficient identification of bioactive hit compounds.

Small molecules that prevent the cell binding of exotoxins have been identified both using un-biased high-content screening exercises as well as high resolution structure-based rational drug design. One notable study utilized an imaging-based phenotypic screen to identify small molecules that protected the cells from *C. difficile* TcdB-induced morphological alterations [141]. The screen lead into identification of methyl cholate, a bile acid derivative. Subsequent validation experiments on binding with differential scanning fluorimetry (DSF) demonstrated that the thermal stability of TcdB was strongly increased by methyl cholate [141]. At cellular level, methyl cholate lowered the amounts of cell-associated TcdB [141]. In an *in vitro* biochemical assay, methyl cholate supressed the IP_6-induced auto-processing activity of TcdB. The data indicates that methyl cholate directly binds to TcdB and induces a conformational change affecting receptor binding and autoprocessing activity. A structure

activity relationship (SAR) study will be very interesting to understand the methyl cholate mode of action.

The cytolytic process of pore-forming toxins of *S. aureus*, α-toxin and bicomponent leukotoxins, begins with the binding of soluble toxin monomers to a cell surface receptor, where they associate to form a nonlytic, oligomeric pre-pore structure [7]. Finally, the translocation of the prestem regions across the membrane results in the bilayer-spanning β-barrel pore structure and consequent membrane permeabilization and cell lysis[7]. In a recent study, crystal structures revealed evolutionarily conserved phosphatidylcholine-binding mechanisms for LukED, PVL and α-toxin [142]. A phosphatidylcholine mimetic compound n-tetradecylphosphocholine (C14PC) was found to able to significantly reduce the lytic activity of these toxins *in vitro*. In addition to broad-spectrum inhibitory action towards LukED, PVL, and α-toxin, C14PC also has low production costs, and thus it might serve as starting-point in the development of agents that reduce the virulence of *S. aureus* infection prophylactically and therapeutically. The C14P compound is also expected to be well-tolerated by humans, as similarly structured drug miltefosine (hexadecylophosphocline C16P, also known as Impavido) is FDA-approved as an oral antiparasitic for the treatment of leishmaniasis [143].

DRUGGABLE STEP II - INTRACELLULAR MATURATION

Intracellular-targeting toxins such as pertussis toxin and anthrax toxin undergo complex maturation process, often involving complete retrograde trafficking from the endosome to Golgi and ER followed by effector subunit release into the cytosol. Exotoxins may rely on their auto-processing properties, e.g. TcdB of *C. difficile*, or be dependent on oligomerization in order to deliver their enzymatic cargo into the cytosol, e.g. anthrax toxin. Antibodies, antibody fragments and small molecules have been identified that interfere with these processes.

MONOCLONAL ANTIBODIES

Large amounts of exotoxin-neutralizing mAbs haven identified (see above, Table 1, Suppl. Table 1). Depending on the binding epitope, these mAbs may not necessarily prevent exotoxin binding to the host cell surface receptor, but act more downstream in the functional pathway of exotoxins (Figure 1). The same applies for the antibody-fragments. The downstream effect is exemplified in the case of developmental pipeline with humanized mAbs PA-50 and PA-41 targeting *C. difficile* TcdA and

TcdB, respectively [144]. The humanized mAbs PA-50 and PA-41 efficiently neutralized TcdA/TcdB in cell culture experiments, and demonstrated efficacy in hamster model for CDI [144]. The PA50 mAb was shown to bind to multiple sites on the TcdA C-terminal combined repetitive oligopeptide (CROP) domain, based on high resolution structural data [145]. Binding of TcdA to the host cell surface was prevented by PA50 mAb indicating that receptor blockade is the mode of action by which PA50 neutralizes TcdA.[145] This is the same mode-of-action how the clinical use bezlotoxoumab (anti-TcdB mAb) and aclotoxumab (anti-TcdA mAb) appear to be working [48-51]. In contrast, an entirely different neutralization mechanism was shown for PA41, the TcdB specific mAb, based on high resolution structural data [146]. The PA41 mAb recognizes a single, highly conserved epitope on the TcdB glucosyltransferase domain [146]. The PA41 mAb does not block TcdB from binding or entering the host cell via endocytosis [146]. The PA41 mAb rather prevents the translocation of the glucosyltransferase enzymatic cargo from the endosome into the host cell cytosol [146]. Alternative mode of actions have also been reported for anthrax toxin-neutralizing mAbs. Following endocytosis of the prepore-EF/LF complex, an acid-driven prepore-to-pore conversion occurs, thus promoting the entry of EF/LF into the cytosol, where they exert their toxic effects [147]. The cAb29, an anti-PA antibody, appeared to prevent the PA-formed pre-pore to undergo conformational changes into the mature pore structure in the acidic endosomal compartment and therefore prevented the toxin cargo delivery into the cytosol [147]. This mode of action is in contrast to obiltoxaximab and raxibacumab, which recognize the receptor-binding region of PA [35, 36] and thereby block PA-host cell surface interactions. Moreover, intracellular maturation blocking mAbs have been identified in the Shiga toxin-focused drug development efforts, e.g. also in the Shigamabs developmental pipeline (see above). For example, human mAb 5C12, which binds to the catalytic A-subunit, did not interfere with the cell surface binding of Stx-2 [148]. In contrast, 5C12 blocked the retrograde transport of Stx-2 into the Golgi and ER, preventing the entry of A-subunit into the cytosol [148]. The 5C12 study demonstrates an important point in respect of the use of exotoxin-neutralizing mAbs. The 5C12 was able to bind to the already cell-bound Stx-2 [148]. This potentially extends the therapeutic window as compared to mAbs, which prevent the cell binding of exotoxins.

SMALL MOLECULES

Interesting development pipelines have been focused on small molecules that interfere with the intracellular maturation of exotoxins, in particular their auto-processing activity. Ebselen (2-phenyl-1,2-benzoselenazol-3-one) is a lipid soluble membrane-penetrating organoselenium compound [149]. Ebselen has generic antioxidant properties, e.g. it catalyzes the reduction of reactive oxygen species

(ROS) in a manner similar to glutathione peroxidase [149]. Ebselen also readily and covalently modify cysteine residues [149]. Ebselen was identified as an inhibitor of the auto-processing cysteine protease domain (CPD) of TcdB in an *in vitro* fluorescence polarization high throughput screen [150]. Follow-up studies demonstrated that Ebselen also inhibited auto-processing of TcdA [150]. Mechanistically, it was shown that Ebselen covalently modified the CPD domain of TcdA/TdB at cysteine residues leading to suppression of cysteine protease activity [150]. Ebselen was also identified independently as TcdB inhibitor in a high throughput cell phenotypic screen [141]. The authors worked out on the mode of action *in vitro*, and proposed, in contrast to [150, 151], that Ebselen acts on the glycosyltransferase activity of TcdB preventing glycosylation of the small GTPase Rac1[152]. The inhibitory action on TcdB appeared to be indirect, acting via Ebselen-mediated modification of cysteine residues on Rac1[152]. The initial screening studies showed that Ebselen protected cells and mice against TcdA/TcdB-mediated killing and improved histopathology in a murine CDI model [141, 150]. Recently, animal experimentation was extended to show that Ebselen, as a monotherapy, reduces recurrence rates and decreases the severity of colitis in animal models of CDI [153]. Moreover, Ebselen has already advanced to phase III clinical trials in diseases unrelated to CDI, e.g. diabetes (NCT00762671). Therefore, Ebselen appears as a well-tolerated drug candidate to treat CDI. As for now, it remains unknown to what extent Ebselen functions via its generic anti-inflammatory properties and to what extent via its anti-TcdA/TcdB functions. Pan-reactivity with cysteine residues is a concerning fact, but the exotoxin neutralization potency itself, not the detailed mechanism of action, is perhaps of more practical interest.

The multifunctional auto-processing repeats-in-toxins (MARTX) toxin, e.g. in *V. cholerae*, also relies on proteolytic auto-processing for cellular activity [154]. Similar to the CPD domains of clostridial toxins TcdA and TcdB, MARTX toxin of *V. cholerae* is activated by IP6 [154]. Covalent cysteine protease inhibitors were identified, which interfered with the MARTX toxin auto-processing [154]. Notably, a high resolution structure CPD in complex with the aza-leucine epoxide inhibitor JCP598 was determined [154]. The overall structure of inhibitor-bound, activated CPD is nearly identical to the activated CPD [154]. The data indicates that the inhibitor docks into the active site cleft created upon binding of IP6 to the CPD [154]. Similar kind of a study has been published on covalent *C. difficile* CPD inhibitors [155], building in part on the work on *V. cholerae* MARTX toxin [154]. High resolution structural information was obtained of the inhibitor-CPD complex and some of the analyzed small molecules were potent in living cells to inhibit TcdB functions [155]. It remains to be determined if the specificity

of these particular covalent protease inhibitors for MARTX and TcdA/TcdB toxins is high enough at the cellular and whole body level to allow further development as a drug lead.

A novel therapeutic paradigm explored the possibility to target the autoproteolysis activity of TcdB by triggering its IP6-induced auto-proteolysis in the gut lumen [156]. To reach this goal, a gain-of-function small molecules, IP6 analogues were synthetized by progressively replacing the IP6 phosphate groups with sulfate groups. This was done in order to reduce the susceptibility of IP6 to complexation at physiological calcium concentrations at colon lumen, while maintaining the uniquely high charge density that mediates its interaction with TcdB. Partial replacement of phosphates by sulfates and thiophosphates resulted in analogs (IP2S4, IT2S4) capable of inducing TcdB cleavage at micromolar concentrations in the presence of calcium [156]. In mouse model of colitis, oral administration of IP2S4, was shown to attenuate the symptoms. Furthermore, treatment with the thiophosphate analog IT2S4, which has improved stability toward inositol phosphatase enzymes that may be present in the gut lumen, rescued mice in the acute CDI model [156]. Taken together, pharmaceutical targeting of the auto-proteolytic activity of exotoxins appears as a very promising therapeutic strategy.

DRUGGABLE STEP III - CYTOSOLIC EFFECTOR FUNCTIONS

This step in the functional pathway of exotoxins refers to the point where the exotoxin, in particular its effector domain, has been released from the endosome or the Golgi/ER compartment into the cytosol. Some exotoxins also gain access into the cytosol straight from the plasma membrane. For instance, NAD+ glycohydrolase (SPN) of *Streptococcus pyogenes* utilizes the multimeric pore structure created by another exotoxin of *S. pyogenes*, streptolysin S (SLO), at the host cell membrane [157]. Also, the bifunctional hemolysin/adenylate cyclase (CyaA) of *Bordetella pertussis* first binds to the surface, and subsequently inserts its cyclic AMP (cAMP)-generating catalytic domain into the cytosolic side of the plasma membrane [158].

SMALL MOLECULES

There have been a number of attempts to develop small molecules inhibiting the cytosolic effector functions of exotoxins. Major advantage with these compounds would be that they are capable of preventing exotoxin functions after the exotoxin has been internalized. This mode of action should

open up wider practical possibilities for the drugs, in particular in therapeutic use. One notable high-content screening exercise was undertaken to identify inhibitors of the glucosyltransferase activity of *C. difficile* TcdB [159]. The compounds were screened utilizing a 1536-well fluorescence polarization assay for UDP-glucose hydrolysis activity by the C-terminal glucosyltransferase domain of TcdB [159]. Multiple hits were identified from diverse six million-member compound collection [159]. Hit-to-lead optimization efforts centered around a novel series of benzodiazepinedione-based inhibitors [159, 160]. Optimized compounds have demonstrated good pharmacokinetic profiles in mouse and hamster and have been efficacious in multiple cell culture and animal models of *C. difficile* infection upon oral dosing [159, 161]. We have recently identified small molecules inhibiting the ADP-ribosyltransferase (ART) activity of pertussis toxin [162]. We developed an *in vitro* high throughput-compatible assay to quantify NAD^+ consumption during PtxS1-catalyzed ADP-ribosylation of Gαi *in vitro*. Two inhibitory compounds (NSC228155 and NSC29193) with low micromolar IC_{50}-values were identified in the *in vitro* NAD^+ consumption assay via screening of a focused compound library containing approximately 2000 small molecules. These compounds were also potent in an independent *in vitro* assay monitoring conjugation of ADP-ribose to Gαi. Moreover, the membrane permeable NSC228155 inhibited the pertussis AB_5 holotoxin-catalyzed ADP-ribosylation of Gαi in living human cells with a low micromolar IC_{50}-value. Although NSC228155 was well-tolerated at these low micromolar inhibitory concentrations, we witnessed significant cellular toxicity with NSC228155 upon our titration analyses. We currently employ medicinal chemistry efforts including molecular modeling and protein crystallography in an attempt to design less toxic NCS228155 analogs with additionally increased potency and specificity.

In addition to *B. pertussis*, ART-toxins are key virulence factors of several pathogens such as *C. diphthteria* (diphtheria toxin), *V. cholera* (cholera toxin) and *E. coli* (heat-labile enterotoxin) [14]. Selective targeting and inhibition of their ADP-ribosyltranferase activity holds promise to interfere with disease pathology. Hit compounds inhibiting *P. aeruginosa* ExoA-induced cytotoxicity in yeast and mammalian cell-based assays *in vitro* have been identified [163]. Virtual screening on the crystal structure of a closely related cholic toxin of *V. cholera* was primarily used to design the screened compound library [164]. Hit compounds for ART-toxins of *B. sphaericus*, *C. difficile*, and *C. botulinum* were found via *in vitro* screening of kinase inhibitors, which are typically adenosine mimics and thereby chemically related to NAD^+ [165]. Bisubstrate analogs mimicking the nicotinamide portion of NAD^+ and arginine residue of the target host cell protein have also been developed to inhibit cholera toxin [166]. In addition, structures of NAD^+- or hit compound-bound ART-toxins have allowed

computational analyses to understand the binding modes and to provide rational ideas for further improvements, as in the case of cholix-toxin of *V. cholera* [163, 167]. However, despite the recent advancements, a rationally designed small molecule targeting bacterial ART-toxins has yet to reach preclinical animal experimentation.

Small molecules that prevent the cytosolic effector functions have also been identified by cell-based screening exercises. The naturally occurring flavonoid phloretin was identified as a compound protecting cells from both *C. difficile* TcdA- and TcdB-induced cell rounding [141]. Subsequent validation experiments demonstrated that phloretin was a direct inhibitor of the toxin GTD domains of both TcdA and TcdB rounding [141]. The authors conducted a secondary focused library screening with flavonoid compounds, and identified two potent analogs of phloretin [141]. Phloretin appears to act as a non-competitive inhibitor and thereby with a probable allosteric action. The authors argued that this mode of action may offer high selectivity and specificity over other enzymes that utilize the same substrate, in this case UDP-glucose [141]. This highlights the drawback, for example, in our own ADP-ribosyltransferase studies where we aimed to identify competitive small molecules binding to the NAD+ binding active site of pertussis toxin [162]. These compounds may also interact with the plethora of other NAD+ binding proteins in the cell, such as members of the poly(ADP-ribose)-polymerase (PARP) protein family [168]. It remains to be studied whether or not these off-target-effects are a concern.

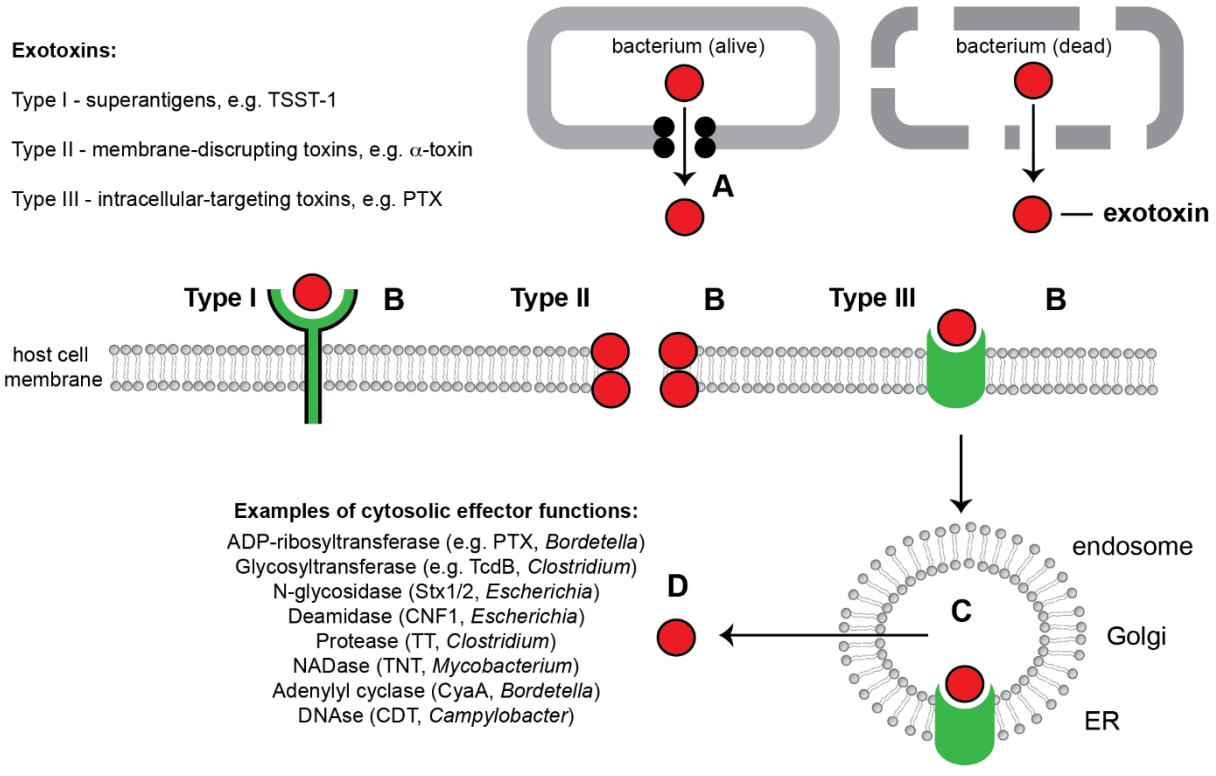
CONCLUSIONS AND FUTURE PERSPECTIVES

The pre-clinical, clinical trial and real-world clinical data demonstrate that exotoxin-targeted antivirulence therapy can be effective, notably exemplified by the toxin B (TcdB)-neutralizing bezlotoxumab to prophylactically reduce recurrence of *C. difficile* infections. Antivirulence drugs have potential as stand-alone prophylactic and therapeutic pharmaceuticals, but they also may complement the use of antibiotics, e.g. to allow lowering of the dosage of antibiotics. Three main reasons are driving the rapid expansion of research on antivirulence therapy. First of all, widespread antibiotic resistance calls for the development of new alternative ways to treat bacterial infections. Secondly, awareness of the physiological importance of microbiota forces us to consider treatment of bacterial infections with more focused pathogen-specific pharmaceuticals. Thirdly, decades of basic research using various *in vitro* assays, cell and tissue culture models and animal experimentation have created an in-depth view on bacterial virulence factors as potential drug targets.

There are a number of challenges to overcome, in particular in the therapeutic use of exotoxin-targeted drug modalities. The therapeutic window is one major concern. Typically, upon clinical suspicion of bacterial infection, patients receive empiric antimicrobial therapy, in many cases broad-spectrum, before we have the diagnostic data. Antivirulence therapy is pathogen-specific, and thereby requires a diagnostic finding to be effective. When such data becomes available, can we still interfere with the disease pathology? The answer appears to be yes, at least based on pre-clinical data with animal experiments on exotoxin-targeted drug modalities. Perhaps the therapeutic window could be extended by directing more pre-clinical development to modalities, which can also act inside the host cell such as small molecules. This modality has the additional benefit that most small molecules can be formulated and optimized for oral administration. This would help the clinical use, including self-medication, as compared to modalities requiring more laborious administration techniques, such as infusion with mAbs. The wider applicability of antivirulence drugs in clinic, in analogy to antibiotics, is another major concern, which also affects the developmental interest of the big pharma. Exotoxins do have a significant and sometimes the primary role as the disease-causing virulence factor, but bacterial virulence is still a multifactorial process. Dominant virulence factors are not even know for many important bacterial pathogens. Perhaps cocktails of different virulence factor-targeting drugs against specific bacterial pathogens could be developed, although this would increase the developmental costs and the length of the developmental pipelines. Good thing is that there is active basic research on exotoxins and other bacterial virulence factors. New virulence factors are being identified. The knowledge on virulence factors prevalence and variability in clinic and nature increases. The high resolution structural understanding of the functional properties of virulence factors is getting better. Taken together, although important progress has been made in the development of exotoxin-targeted drug modalities, and antivirulence therapy, significant work is still required to realize the potential of these promising pharmaceuticals.

ACKOWLEDGEMENTS

The authors apologize to those colleagues whose work and original publications could not be described in this review because of space limitations. The exotoxin research in the laboratory of Arto Pulliainen (A.T.P.) has received financial support from Turku Doctoral Programme of Molecular Medicine (TuDMM) (to Moona Sakari, M.S.), The Finnish Cultural Foundation (to M.S.), Turku University Foundation (to M.S.), Academy of Finland (grant no. 295296, to A.T.P.), Sigrid Juselius Foundation (to A.T.P.), Instruct ERIC (to A.T.P.), The Finnish Society of Sciences and Letters (to A.T.P.) and University of Turku, Turku, Finland (to A.T.P.). The funders have had no role in study designs, data collection and interpretations, or the decisions to submit the results for publication. The authors declare that they have no conflicts of interest.



The main druggable steps in the functional pathway of exotoxins:
A) secretion B) cell surface binding C) intracellular maturation D) cytosolic effector functions

Figure 1. (title on next page)

Figure 1. Exotoxin mode of functions and main druggable steps in the functional pathway of exotoxins. Exotoxins are bacterial proteins that are either actively secreted from the bacterium in an energy-dependent process or they become soluble upon bacterial lysis. Exotoxins recognize the host cell surface via specific receptor structures composed of proteins, lipids or carbohydrates. Exotoxins have potent host modulating activities either at the host cell surface or inside the host cell. Intracellular-targeting toxins undergo complex maturation process, often involving complete retrograde trafficking process from the endosome to Golgi and ER followed by effector subunit release into the cytosol. Exotoxins are typically classified in three different types, i.e. Type I - superantigens, Type II - membrane-disrupting toxins (pore-forming toxins, lipid-modifying enzymes and detergent-like peptides), and Type III - intracellular-targeting toxins. Some overlap exists between these three types, e.g. listeriolysin of *Listeria monocytogenes* forms pores in the endosomal membranes. TSST-1, toxic shock syndrome toxin; PTX, pertussis toxin; TcdB, toxin B; Stx1/2, Shiga toxin 1/2; CNF1, necrotizing factor 1; TT, tetanus toxin; TNT, tuberculosis necrotizing toxin; CyaA, bifunctional hemolysin/adenylyl cyclase; CDT, cytolethal distending toxin.

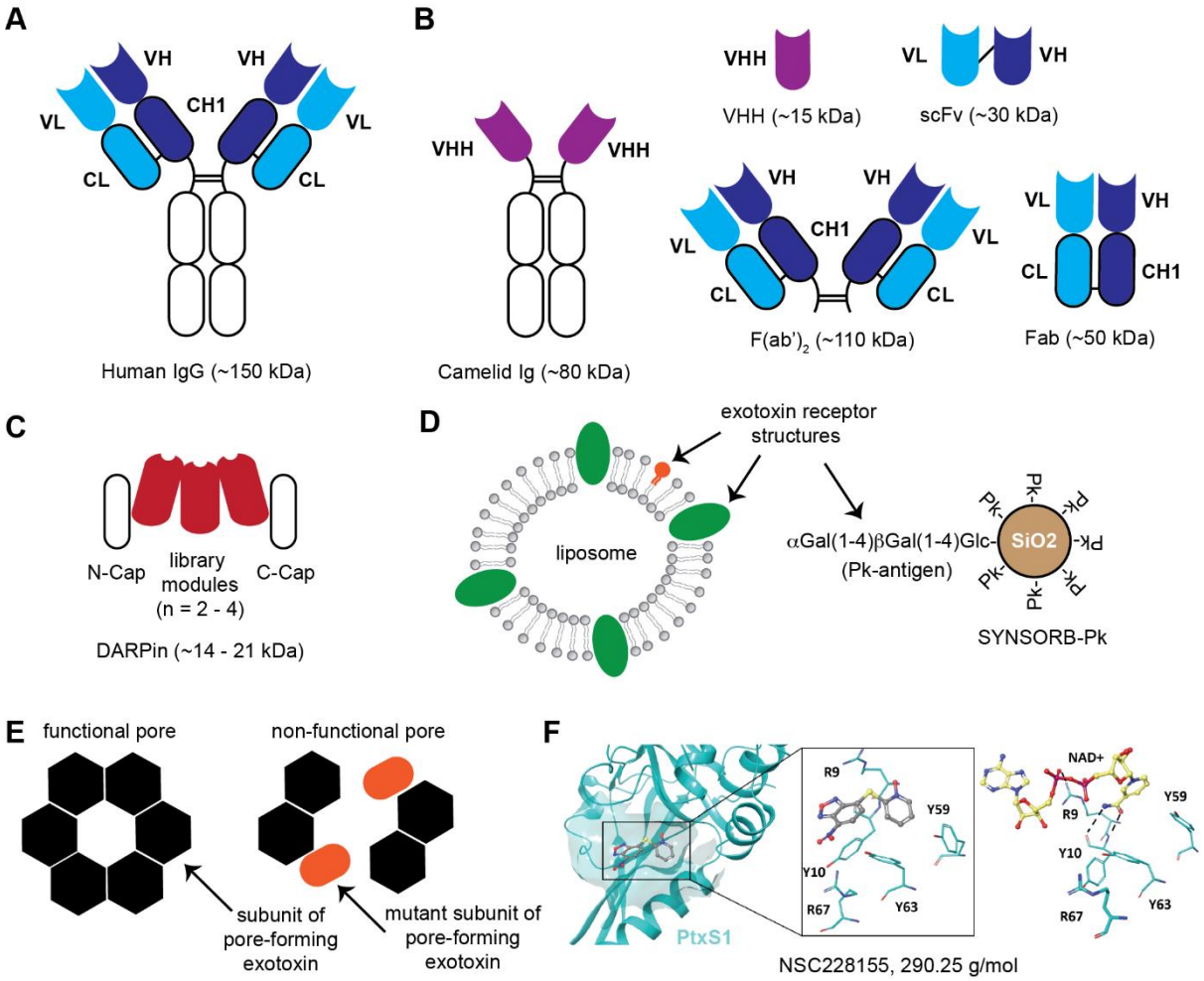


Figure 2. (title on next page)

Figure 2. Exotoxin-targeted drug modalities. Schematic representations of the different exotoxin-targeted drug modalities that are under pre-clinical and clinical development or use. The modalities and their examples are not drawn in scale. **A)** ***Monoclonal antibodies (mAbs).*** The mAbs have several advantages in exotoxin-targeting such as high specificity, long in *vivo* half-life in circulation and good tolerability. All currently FDA-approved exotoxin-neutralizing drugs are mAbs (Table 1). **B)** ***Antibody fragments.*** Antibody fragments can be produced more easily, as compared to mAbs, which results in faster cultivation, higher yields, and lower production costs. Their small size also allows better tissue penetration and they may have reduced immunogenicity. Antibody engineering also utilizes antibody fragments, e.g. linking the most efficient fragments with Fc-region to engage effector functions of entire mAbs. **C)** ***Antibody mimetics.*** Antibody mimetics, such as DARPins, are diverse group of single-domain protein scaffolds that are thermostable, highly engineerable and can be produced in microorganisms or even be completely synthesized chemically. **D)** ***Receptor analogs and neutralizing scaffolds.*** This is a very heterogeneous group of drug candidates, including exotoxin-binding polymeric molecules, e.g. Tolevamer, polyvalent exotoxin receptor analogs, e.g. SYNSORB-Pk, and various liposome-, exosome-, nanoparticle- or even bacteria-based carrier particles having exotoxin-absorbing macromolecules. **E)** ***Dominant negative subunits.*** Exotoxins that functionally rely on multimerization, such as pore-forming toxins, can be trapped to a pre-mature state by dominant negative subunits. One flavor of this approach utilizes peptides derived from the exotoxin subunits that interfere with the assembly process. ***F) Small molecules.*** Small molecules have been the traditional basis for drug development. Due to their small size, small molecules penetrate tissues efficiently, and may also enter the cell allowing effective targeting of intracellular processes, such as the enzymatic functions of exotoxins (Figure 1). As for now, however, a limited amount of small molecule high-throughput screening studies have been conducted on exotoxins. Example taken from our recent *in vitro* screening exercise to identify inhibitors of the ADP-ribosyltransferase activity of pertussis toxin [162]. Refer to the main text, Table 1 and Suppl. Table 1 for more description.

Table 1. Exotoxin-targeted drugs that are either FDA-approved or that have entered clinical trials. Clinical trial data based on ClinicalTrials.gov database as literature searches, as of 18th march 2021 (https://www.clinicaltrials.gov). Ebselen trials have been conducted in diseases other than *C. difficile* infections, e.g. diabetes phase III trial NCT00762671.

mAb	Format	Pathogen	Target	State	Trial ID
Raxibacumab (Abthrax®)	h(human)/IgG1	*B. anthracis*	Anthrax toxin	FDA 2012 Phase IV	NCT00639678[36] NCT02016963 NCT02339155[45] NCT02177721
Obiltoxaximab (Anthim®)	c(chimeric)/ IgG1	*B. anthracis*	Anthrax toxin	FDA 2016 Phase IV	NCT00138411 NCT00829582 NCT01932242[169] NCT01929226[169] NCT01453907[169] NCT01932437 NCT01952444[169] NCT03088111
Bezlotoxumab (Zinplava®)	h/IgG1	*C. difficile*	Toxin B (TcdB)	FDA 2016 Phase IV	NCT01241552[47] NCT01513239[47] NCT04626947 NCT03880539 NCT03937999 NCT03756454 NCT04415918 NCT03182907 NCT03829475 NCT04317963 NCT04075422 NCT04725123
ASN100	2 x h/IgG1	*S. aureus*	α-toxin, 5 leukocidins	Phase II (terminated)	NCT02940626 NCT01357213 [170]
MEDI4893 (Suvratoxumab)	h/IgG1	*S. aureus*	α-toxin	Phase II	NCT02296320 [67]

					NCT01769417
AR-301 (Tosatoxumab)	h/IgG1	*S. aureus*	α-toxin	Phase III	NCT01589185 [68] NCT03816956
Shigamabs	2 x c/IgG1	*E. coli*	Shiga toxin 1-2 (Stx1-2)	Phase II	NCT01252199
TMA-15 (Urtoxazumab)	hIgG1	*E. coli*	Stx2	Phase I	not availabe[80]
XOMA 3Ab	c/IgG1 2 x h/IgG1	*C. botulinum*	Botulinum neurotoxin A (BoNT/A)	Phase I	NCT01357213 [170]
NTM-1632	3 x c/IgG1	*C. botulinum*	BoNT/B	Phase I	NCT02779140
NTM-1634	4 x h/IgG1	*C. botulinum*	BoNT/C-D	Phase I	NCT03046550 [171]
NTM-1633	3 x c/IgG1	*C. botulinum*	BoNT/E	Phase I	NCT03603665
S315	h/IgG1	*C. diphteriae*	Diphtheria toxin	Phase I	NCT04075175

Receptor analog	**Format**	**Pathogen**	**Target**	**Current state**	**Trial ID**
SYNSORB-Pk	polyvalent carbohydrate conjugate	*E. coli*	Stx1-2	Phase III (failed)	NCT00004465[108]

Neuralizing scaffold	**Format**	**Pathogen**	**Target**	**Current state**	**Trial ID**
Tolevamer	styrene sulfonate polymer	*C. difficile*	TcdA-B	Phase III (failed)	NCT00106509[114] NCT00196794[114] NCT00382304 NCT00466635 NCT00034294
CAL02	liposome	*S. pneumoniae*	pneumolysin	Phase I	NCT02583373 [131]

Small molecule	Format	Pathogen	Target	Current state	Trial ID
Ebselen	organoselenium compound	*C. difficile*	TcdA-B	pre-clinical (Phase III)	NCT01452607 NCT00762671

REFERENCES

1. Diard, M.; Hardt, W. D., Evolution of bacterial virulence. *FEMS Microbiol Rev* **2017,** *41* (5), 679-697. DOI: 10.1093/femsre/fux023.
2. Clatworthy, A. E.; Pierson, E.; Hung, D. T., Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* **2007,** *3* (9), 541-8. DOI: 10.1038/nchembio.2007.24.
3. Otshudiema, J. O.; Acosta, A. M.; Cassiday, P. K.; Hadler, S. C.; Hariri, S.; Tiwari, T. S. P., Respiratory illness caused by *Corynebacterium diphtheriae* and *C. ulcerans*, and use of diphtheria anti-toxin in the United States, 1996-2018. *Clin Infect Dis* **2020**. DOI: 10.1093/cid/ciaa1218.
4. Richardson, J. S.; Parrera, G. S.; Astacio, H.; Sahota, H.; Anderson, D. M.; Hall, C.; Babinchak, T., Safety and linical Outcomes of an Equine-derived Heptavalent Botulinum Antitoxin Treatment for Confirmed or Suspected Botulism in the United States. *Clin Infect Dis* **2020,** *70* (9), 1950-1957. DOI: 10.1093/cid/ciz515.
5. Payne, J. R.; Khouri, J. M.; Jewell, N. P.; Arnon, S. S., Efficacy of Human Botulism Immune Globulin for the Treatment of Infant Botulism: The First 12 Years Post Licensure. *J Pediatr* **2018,** *193*, 172-177. DOI: 10.1016/j.jpeds.2017.10.035.
6. Bergsten, H.; Madsen, M. B.; Bergey, F.; Hyldegaard, O.; Skrede, S.; Arnell, P.; Oppegaard, O.; Itzek, A.; Perner, A.; Svensson, M.; Norrby-Teglund, A.; Group, I. S., Correlation Between Immunoglobulin Dose Administered and Plasma Neutralization of Streptococcal Superantigens in Patients With Necrotizing Soft Tissue Infections. *Clin Infect Dis* **2020,** *71* (7), 1772-1775. DOI: 10.1093/cid/ciaa022.
7. Oliveira, D.; Borges, A.; Simões, M., *Staphylococcus aureus* Toxins and Their Molecular Activity in Infectious Diseases. *Toxins (Basel)* **2018,** *10* (6). DOI: 10.3390/toxins10060252.
8. Scanlon, K.; Skerry, C.; Carbonetti, N., Association of Pertussis Toxin with Severe Pertussis Disease. *Toxins (Basel)* **2019,** *11* (7). DOI: 10.3390/toxins11070373.
9. Weiss, A. A.; Johnson, F. D.; Burns, D. L., Molecular characterization of an operon required for pertussis toxin secretion. *Proc Natl Acad Sci U S A* **1993,** *90* (7), 2970-4.
10. Stein, P. E.; Boodhoo, A.; Armstrong, G. D.; Cockle, S. A.; Klein, M. H.; Read, R. J., The crystal structure of pertussis toxin. *Structure* **1994,** *2* (1), 45-57.
11. Stein, P. E.; Boodhoo, A.; Armstrong, G. D.; Heerze, L. D.; Cockle, S. A.; Klein, M. H.; Read, R. J., Structure of a pertussis toxin-sugar complex as a model for receptor binding. *Nat Struct Biol* **1994,** *1* (9), 591-6.
12. Plaut, R. D.; Carbonetti, N. H., Retrograde transport of pertussis toxin in the mammalian cell. *Cell Microbiol* **2008,** *10* (5), 1130-9. DOI: 10.1111/j.1462-5822.2007.01115.x.
13. Hazes, B.; Boodhoo, A.; Cockle, S. A.; Read, R. J., Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. *J Mol Biol* **1996,** *258* (4), 661-71. DOI: 10.1006/jmbi.1996.0277.
14. Simon, N. C.; Aktories, K.; Barbieri, J. T., Novel bacterial ADP-ribosylating toxins: structure and function. *Nat Rev Microbiol* **2014,** *12* (9), 599-611. DOI: 10.1038/nrmicro3310.

15. Banerjee, T.; Cilenti, L.; Taylor, M.; Showman, A.; Tatulian, S. A.; Teter, K., Thermal Unfolding of the Pertussis Toxin S1 Subunit Facilitates Toxin Translocation to the Cytosol by the Mechanism of Endoplasmic Reticulum-Associated Degradation. *Infect Immun* **2016,** *84* (12), 3388-3398. DOI: 10.1128/IAI.00732-16.
16. Katada, T.; Ui, M., Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc Natl Acad Sci U S A* **1982,** *79* (10), 3129-33.
17. West, R. E.; Moss, J.; Vaughan, M.; Liu, T.; Liu, T. Y., Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. *J Biol Chem* **1985,** *260* (27), 14428-30.
18. Graf, R.; Codina, J.; Birnbaumer, L., Peptide inhibitors of ADP-ribosylation by pertussis toxin are substrates with affinities comparable to those of the trimeric GTP-binding proteins. *Mol Pharmacol* **1992,** *42* (5), 760-4.
19. Paramonov, V. M.; Sahlgren, C.; Rivero-Müller, A.; Pulliainen, A. T., iGIST-A Kinetic Bioassay for Pertussis Toxin Based on Its Effect on Inhibitory GPCR Signaling. *ACS Sens* **2020**. DOI: 10.1021/acssensors.0c01340.
20. Jin, J.; Hsieh, Y. H.; Cui, J.; Damera, K.; Dai, C.; Chaudhary, A. S.; Zhang, H.; Yang, H.; Cao, N.; Jiang, C.; Vaara, M.; Wang, B.; Tai, P. C., Using Chemical Probes to Assess the Feasibility of Targeting SecA for Developing Antimicrobial Agents against Gram-Negative Bacteria. *ChemMedChem* **2016,** *11* (22), 2511-2521. DOI: 10.1002/cmdc.201600421.
21. Feng, C.; Huang, Y.; He, W.; Cheng, X.; Liu, H.; Ma, B.; Zhang, W.; Liao, C.; Wu, W.; Shao, Y.; Xu, D.; Su, Z.; Lu, W., Tanshinones: First-in-Class Inhibitors of the Biogenesis of the Type 3 Secretion System Needle of *Pseudomonas aeruginosa* for Antibiotic Therapy. *ACS Cent Sci* **2019,** *5* (7), 1278-1288. DOI: 10.1021/acscentsci.9b00452.
22. DiGiandomenico, A.; Keller, A. E.; Gao, C.; Rainey, G. J.; Warrener, P.; Camara, M. M.; Bonnell, J.; Fleming, R.; Bezabeh, B.; Dimasi, N.; Sellman, B. R.; Hilliard, J.; Guenther, C. M.; Datta, V.; Zhao, W.; Yu, X. Q.; Suzich, J. A.; Stover, C. K., A multifunctional bispecific antibody protects against *Pseudomonas aeruginosa*. *Sci Transl Med* **2014,** *6* (262), 262ra155. DOI: 10.1126/scitranslmed.3009655.
23. Jain, R.; Beckett, V. V.; Konstan, M. W.; Accurso, F. J.; Burns, J. L.; Mayer-Hamblett, N.; Milla, C.; VanDevanter, D. R.; Chmiel, J. F.; Group, K.-A. S., KB001-A, a novel anti-inflammatory, found to be safe and well-tolerated in cystic fibrosis patients infected with *Pseudomonas aeruginosa*. *J Cyst Fibros* **2018,** *17* (4), 484-491. DOI: 10.1016/j.jcf.2017.12.006.
24. Tam, J.; Hamza, T.; Ma, B.; Chen, K.; Beilhartz, G. L.; Ravel, J.; Feng, H.; Melnyk, R. A., Host-targeted niclosamide inhibits *C. difficile* virulence and prevents disease in mice without disrupting the gut microbiota. *Nat Commun* **2018,** *9* (1), 5233. DOI: 10.1038/s41467-018-07705-w.
25. Stechmann, B.; Bai, S. K.; Gobbo, E.; Lopez, R.; Merer, G.; Pinchard, S.; Panigai, L.; Tenza, D.; Raposo, G.; Beaumelle, B.; Sauvaire, D.; Gillet, D.; Johannes, L.; Barbier, J., Inhibition of retrograde transport protects mice from lethal ricin challenge. *Cell* **2010,** *141* (2), 231-42. DOI: 10.1016/j.cell.2010.01.043.
26. Secher, T.; Shima, A.; Hinsinger, K.; Cintrat, J. C.; Johannes, L.; Barbier, J.; Gillet, D.; Oswald, E., Retrograde Trafficking Inhibitor of Shiga Toxins Reduces Morbidity and Mortality of Mice Infected with Enterohemorrhagic *Escherichia coli*. *Antimicrob Agents Chemother* **2015,** *59* (8), 5010-3. DOI: 10.1128/AAC.00455-15.
27. Hartmann, S.; Lopez Cruz, R.; Alameh, S.; Ho, C. C.; Rabideau, A.; Pentelute, B. L.; Bradley, K. A.; Martchenko, M., Characterization of Novel Piperidine-Based Inhibitor of Cathepsin B-Dependent Bacterial Toxins and Viruses. *ACS Infect Dis* **2018,** *4* (8), 1235-1245. DOI: 10.1021/acsinfecdis.8b00053.
28. Gillespie, E. J.; Ho, C. L.; Balaji, K.; Clemens, D. L.; Deng, G.; Wang, Y. E.; Elsaesser, H. J.; Tamilselvam, B.; Gargi, A.; Dixon, S. D.; France, B.; Chamberlain, B. T.; Blanke, S. R.; Cheng, G.; de la Torre, J. C.; Brooks, D. G.; Jung, M. E.; Colicelli, J.; Damoiseaux, R.; Bradley, K. A., Selective inhibitor of endosomal trafficking pathways exploited by multiple toxins and viruses. *Proc Natl Acad Sci U S A* **2013,** *110* (50), E4904-12. DOI: 10.1073/pnas.1302334110.
29. Ernst, K.; Mittler, A. K.; Winkelmann, V.; Kling, C.; Eberhardt, N.; Anastasia, A.; Sonnabend, M.; Lochbaum, R.; Wirsching, J.; Sakari, M.; Pulliainen, A. T.; Skerry, C.; Carbonetti, N. H.; Frick, M.;

Barth, H., Pharmacological targeting of host chaperones protects from pertussis toxin *in vitro* and *in vivo*. *Sci Rep* **2021,** *11* (1), 5429. DOI: 10.1038/s41598-021-84817-2.
30. Chiu, M. L.; Gilliland, G. L., Engineering antibody therapeutics. *Curr Opin Struct Biol* **2016,** *38*, 163-73. DOI: 10.1016/j.sbi.2016.07.012.
31. Moayeri, M.; Leppla, S. H.; Vrentas, C.; Pomerantsev, A. P.; Liu, S., Anthrax Pathogenesis. *Annu Rev Microbiol* **2015,** *69*, 185-208. DOI: 10.1146/annurev-micro-091014-104523.
32. Mohamed, N.; Clagett, M.; Li, J.; Jones, S.; Pincus, S.; D'Alia, G.; Nardone, L.; Babin, M.; Spitalny, G.; Casey, L., A high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized *Bacillus anthracis* spore challenge. *Infect Immun* **2005,** *73* (2), 795-802. DOI: 10.1128/IAI.73.2.795-802.2005.
33. Little, S. F.; Leppla, S. H.; Cora, E., Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. *Infect Immun* **1988,** *56* (7), 1807-13. DOI: 10.1128/IAI.56.7.1807-1813.1988.
34. Maynard, J. A.; Maassen, C. B.; Leppla, S. H.; Brasky, K.; Patterson, J. L.; Iverson, B. L.; Georgiou, G., Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. *Nat Biotechnol* **2002,** *20* (6), 597-601. DOI: 10.1038/nbt0602-597.
35. Leysath, C. E.; Monzingo, A. F.; Maynard, J. A.; Barnett, J.; Georgiou, G.; Iverson, B. L.; Robertus, J. D., Crystal structure of the engineered neutralizing antibody M18 complexed to domain 4 of the anthrax protective antigen. *J Mol Biol* **2009,** *387* (3), 680-93. DOI: 10.1016/j.jmb.2009.02.003.
36. Migone, T. S.; Subramanian, G. M.; Zhong, J.; Healey, L. M.; Corey, A.; Devalaraja, M.; Lo, L.; Ullrich, S.; Zimmerman, J.; Chen, A.; Lewis, M.; Meister, G.; Gillum, K.; Sanford, D.; Mott, J.; Bolmer, S. D., Raxibacumab for the treatment of inhalational anthrax. *N Engl J Med* **2009,** *361* (2), 135-44. DOI: 10.1056/NEJMoa0810603.
37. Nagy, C. F.; Mondick, J.; Serbina, N.; Casey, L. S.; Carpenter, S. E.; French, J.; Guttendorf, R., Animal-to-Human Dose Translation of Obiltoxaximab for Treatment of Inhalational Anthrax Under the US FDA Animal Rule. *Clin Transl Sci* **2017,** *10* (1), 12-19. DOI: 10.1111/cts.12433.
38. Subramanian, G. M.; Cronin, P. W.; Poley, G.; Weinstein, A.; Stoughton, S. M.; Zhong, J.; Ou, Y.; Zmuda, J. F.; Osborn, B. L.; Freimuth, W. W., A phase 1 study of PAmAb, a fully human monoclonal antibody against *Bacillus anthracis* protective antigen, in healthy volunteers. *Clin Infect Dis* **2005,** *41* (1), 12-20. DOI: 10.1086/430708.
39. Cui, X.; Li, Y.; Moayeri, M.; Choi, G. H.; Subramanian, G. M.; Li, X.; Haley, M.; Fitz, Y.; Feng, J.; Banks, S. M.; Leppla, S. H.; Eichacker, P. Q., Late treatment with a protective antigen-directed monoclonal antibody improves hemodynamic function and survival in a lethal toxin-infused rat model of anthrax sepsis. *J Infect Dis* **2005,** *191* (3), 422-34. DOI: 10.1086/427189.
40. Yamamoto, B. J.; Shadiack, A. M.; Carpenter, S.; Sanford, D.; Henning, L. N.; Gonzales, N.; O'Connor, E.; Casey, L. S.; Serbina, N. V., Obiltoxaximab Prevents Disseminated *Bacillus anthracis* Infection and Improves Survival during Pre- and Postexposure Prophylaxis in Animal Models of Inhalational Anthrax. *Antimicrob Agents Chemother* **2016,** *60* (10), 5796-805. DOI: 10.1128/AAC.01102-16.
41. Yamamoto, B. J.; Shadiack, A. M.; Carpenter, S.; Sanford, D.; Henning, L. N.; O'Connor, E.; Gonzales, N.; Mondick, J.; French, J.; Stark, G. V.; Fisher, A. C.; Casey, L. S.; Serbina, N. V., Efficacy Projection of Obiltoxaximab for Treatment of Inhalational Anthrax across a Range of Disease Severity. *Antimicrob Agents Chemother* **2016,** *60* (10), 5787-95. DOI: 10.1128/AAC.00972-16.
42. Corey, A.; Migone, T. S.; Bolmer, S.; Fiscella, M.; Ward, C.; Chen, C.; Meister, G., *Bacillus anthracis* protective antigen kinetics in inhalation spore-challenged untreated or levofloxacin/raxibacumab-treated New Zealand white rabbits. *Toxins (Basel)* **2013,** *5* (1), 120-38. DOI: 10.3390/toxins5010120.
43. Biron, B.; Beck, K.; Dyer, D.; Mattix, M.; Twenhafel, N.; Nalca, A., Efficacy of ETI-204 monoclonal antibody as an adjunct therapy in a New Zealand white rabbit partial survival model for inhalational anthrax. *Antimicrob Agents Chemother* **2015,** *59* (4), 2206-14. DOI: 10.1128/AAC.04593-14.
44. Remy, K. E.; Cui, X.; Li, Y.; Sun, J.; Solomon, S. B.; Fitz, Y.; Barochia, A. V.; Al-Hamad, M.; Moayeri, M.; Leppla, S. H.; Eichacker, P. Q., Raxibacumab augments hemodynamic support and improves outcomes during shock with *B. anthracis* edema toxin alone or together with lethal toxin in canines. *Intensive Care Med Exp* **2015,** *3* (1), 9. DOI: 10.1186/s40635-015-0043-4.
45. Skoura, N.; Wang-Jairaj, J.; Della Pasqua, O.; Chandrasekaran, V.; Billiard, J.; Yeakey, A.; Smith, W.; Steel, H.; Tan, L. K., Effect of raxibacumab on immunogenicity of Anthrax Vaccine Adsorbed: a phase

4, open-label, parallel-group, randomised non-inferiority study. *Lancet Infect Dis* **2020,** *20* (8), 983-991. DOI: 10.1016/S1473-3099(20)30069-4.
46. Smits, W. K.; Lyras, D.; Lacy, D. B.; Wilcox, M. H.; Kuijper, E. J., *Clostridium difficile* infection. *Nat Rev Dis Primers* **2016,** *2*, 16020. DOI: 10.1038/nrdp.2016.20.
47. Wilcox, M. H.; Gerding, D. N.; Poxton, I. R.; Kelly, C.; Nathan, R.; Birch, T.; Cornely, O. A.; Rahav, G.; Bouza, E.; Lee, C.; Jenkin, G.; Jensen, W.; Kim, Y. S.; Yoshida, J.; Gabryelski, L.; Pedley, A.; Eves, K.; Tipping, R.; Guris, D.; Kartsonis, N.; Dorr, M. B.; Investigators, M. I. a. M. I., Bezlotoxumab for Prevention of Recurrent *Clostridium difficile* Infection. *N Engl J Med* **2017,** *376* (4), 305-317. DOI: 10.1056/NEJMoa1602615.
48. Babcock, G. J.; Broering, T. J.; Hernandez, H. J.; Mandell, R. B.; Donahue, K.; Boatright, N.; Stack, A. M.; Lowy, I.; Graziano, R.; Molrine, D.; Ambrosino, D. M.; Thomas, W. D., Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. *Infect Immun* **2006,** *74* (11), 6339-47. DOI: 10.1128/IAI.00982-06.
49. Orth, P.; Xiao, L.; Hernandez, L. D.; Reichert, P.; Sheth, P. R.; Beaumont, M.; Yang, X.; Murgolo, N.; Ermakov, G.; DiNunzio, E.; Racine, F.; Karczewski, J.; Secore, S.; Ingram, R. N.; Mayhood, T.; Strickland, C.; Therien, A. G., Mechanism of action and epitopes of *Clostridium difficile* toxin B-neutralizing antibody bezlotoxumab revealed by X-ray crystallography. *J Biol Chem* **2014,** *289* (26), 18008-21. DOI: 10.1074/jbc.M114.560748.
50. Gupta, P.; Zhang, Z.; Sugiman-Marangos, S. N.; Tam, J.; Raman, S.; Julien, J. P.; Kroh, H. K.; Lacy, D. B.; Murgolo, N.; Bekkari, K.; Therien, A. G.; Hernandez, L. D.; Melnyk, R. A., Functional defects in *Clostridium difficile* TcdB toxin uptake identify CSPG4 receptor-binding determinants. *J Biol Chem* **2017,** *292* (42), 17290-17301. DOI: 10.1074/jbc.M117.806687.
51. Hernandez, L. D.; Kroh, H. K.; Hsieh, E.; Yang, X.; Beaumont, M.; Sheth, P. R.; DiNunzio, E.; Rutherford, S. A.; Ohi, M. D.; Ermakov, G.; Xiao, L.; Secore, S.; Karczewski, J.; Racine, F.; Mayhood, T.; Fischer, P.; Sher, X.; Gupta, P.; Lacy, D. B.; Therien, A. G., Epitopes and Mechanism of Action of the *Clostridium difficile* Toxin A-Neutralizing Antibody Actoxumab. *J Mol Biol* **2017,** *429* (7), 1030-1044. DOI: 10.1016/j.jmb.2017.02.010.
52. Hernandez, L. D.; Racine, F.; Xiao, L.; DiNunzio, E.; Hairston, N.; Sheth, P. R.; Murgolo, N. J.; Therien, A. G., Broad coverage of genetically diverse strains of *Clostridium difficile* by actoxumab and bezlotoxumab predicted by *in vitro* neutralization and epitope modeling. *Antimicrob Agents Chemother* **2015,** *59* (2), 1052-60. DOI: 10.1128/AAC.04433-14.
53. Yang, Z.; Ramsey, J.; Hamza, T.; Zhang, Y.; Li, S.; Yfantis, H. G.; Lee, D.; Hernandez, L. D.; Seghezzi, W.; Furneisen, J. M.; Davis, N. M.; Therien, A. G.; Feng, H., Mechanisms of protection against *Clostridium difficile* infection by the monoclonal antitoxin antibodies actoxumab and bezlotoxumab. *Infect Immun* **2015,** *83* (2), 822-31. DOI: 10.1128/IAI.02897-14.
54. Lowy, I.; Molrine, D. C.; Leav, B. A.; Blair, B. M.; Baxter, R.; Gerding, D. N.; Nichol, G.; Thomas, W. D.; Leney, M.; Sloan, S.; Hay, C. A.; Ambrosino, D. M., Treatment with monoclonal antibodies against *Clostridium difficile* toxins. *N Engl J Med* **2010,** *362* (3), 197-205. DOI: 10.1056/NEJMoa0907635.
55. Johnson, S.; Citron, D. M.; Gerding, D. N.; Wilcox, M. H.; Goldstein, E. J. C.; Sambol, S. P.; Best, E. L.; Eves, K.; Jansen, E.; Dorr, M. B., Efficacy of bezlotoxumab in trial participants infected with *Clostridioides difficile* strain BI associated with poor outcomes. *Clin Infect Dis* **2020**. DOI: 10.1093/cid/ciaa1035.
56. Goldstein, E. J. C.; Citron, D. M.; Gerding, D. N.; Wilcox, M. H.; Gabryelski, L.; Pedley, A.; Zeng, Z.; Dorr, M. B., Bezlotoxumab for the Prevention of Recurrent *Clostridioides difficile* Infection: 12-Month Observational Data From the Randomized Phase III Trial, MODIFY II. *Clin Infect Dis* **2020,** *71* (4), 1102-1105. DOI: 10.1093/cid/ciz1151.
57. Oksi, J.; Aalto, A.; Säilä, P.; Partanen, T.; Anttila, V. J.; Mattila, E., Real-world efficacy of bezlotoxumab for prevention of recurrent *Clostridium difficile* infection: a retrospective study of 46 patients in five university hospitals in Finland. *Eur J Clin Microbiol Infect Dis* **2019,** *38* (10), 1947-1952. DOI: 10.1007/s10096-019-03630-y.
58. Prabhu, V. S.; Dubberke, E. R.; Dorr, M. B.; Elbasha, E.; Cossrow, N.; Jiang, Y.; Marcella, S., Cost-effectiveness of Bezlotoxumab Compared With Placebo for the Prevention of Recurrent *Clostridium difficile* Infection. *Clin Infect Dis* **2018,** *66* (3), 355-362. DOI: 10.1093/cid/cix809.

59. Lee, A. S.; de Lencastre, H.; Garau, J.; Kluytmans, J.; Malhotra-Kumar, S.; Peschel, A.; Harbarth, S., Methicillin-resistant *Staphylococcus aureus*. *Nat Rev Dis Primers* **2018,** *4*, 18033. DOI: 10.1038/nrdp.2018.33.
60. Badarau, A.; Rouha, H.; Malafa, S.; Battles, M. B.; Walker, L.; Nielson, N.; Dolezilkova, I.; Teubenbacher, A.; Banerjee, S.; Maierhofer, B.; Weber, S.; Stulik, L.; Logan, D. T.; Welin, M.; Mirkina, I.; Pleban, C.; Zauner, G.; Gross, K.; Jägerhofer, M.; Magyarics, Z.; Nagy, E., Context matters: The importance of dimerization-induced conformation of the LukGH leukocidin of *Staphylococcus aureus* for the generation of neutralizing antibodies. *MAbs* **2016,** *8* (7), 1347-1360. DOI: 10.1080/19420862.2016.1215791.
61. Rouha, H.; Badarau, A.; Visram, Z. C.; Battles, M. B.; Prinz, B.; Magyarics, Z.; Nagy, G.; Mirkina, I.; Stulik, L.; Zerbs, M.; Jägerhofer, M.; Maierhofer, B.; Teubenbacher, A.; Dolezilkova, I.; Gross, K.; Banerjee, S.; Zauner, G.; Malafa, S.; Zmajkovic, J.; Maier, S.; Mabry, R.; Krauland, E.; Wittrup, K. D.; Gerngross, T. U.; Nagy, E., Five birds, one stone: neutralization of α-hemolysin and 4 bi-component leukocidins of *Staphylococcus aureus* with a single human monoclonal antibody. *MAbs* **2015,** *7* (1), 243-54. DOI: 10.4161/19420862.2014.985132.
62. Rouha, H.; Weber, S.; Janesch, P.; Maierhofer, B.; Gross, K.; Dolezilkova, I.; Mirkina, I.; Visram, Z. C.; Malafa, S.; Stulik, L.; Badarau, A.; Nagy, E., Disarming *Staphylococcus aureus* from destroying human cells by simultaneously neutralizing six cytotoxins with two human monoclonal antibodies. *Virulence* **2018,** *9* (1), 231-247. DOI: 10.1080/21505594.2017.1391447.
63. Stulik, L.; Rouha, H.; Labrousse, D.; Visram, Z. C.; Badarau, A.; Maierhofer, B.; Groß, K.; Weber, S.; Kramarić, M. D.; Glojnarić, I.; Nagy, G.; Croisier, D.; Nagy, E., Preventing lung pathology and mortality in rabbit *Staphylococcus aureus* pneumonia models with cytotoxin-neutralizing monoclonal IgGs penetrating the epithelial lining fluid. *Sci Rep* **2019,** *9* (1), 5339. DOI: 10.1038/s41598-019-41826-6.
64. Magyarics, Z.; Leslie, F.; Bartko, J.; Rouha, H.; Luperchio, S.; Schörgenhofer, C.; Schwameis, M.; Derhaschnig, U.; Lagler, H.; Stiebellehner, L.; Firbas, C.; Weber, S.; Campanaro, E.; Jilma, B.; Nagy, E.; Stevens, C., Randomized, Double-Blind, Placebo-Controlled, Single-Ascending-Dose Study of the Penetration of a Monoclonal Antibody Combination (ASN100) Targeting *Staphylococcus aureus* Cytotoxins in the Lung Epithelial Linig Fluid of HEalthy Volunteers. *Antimicrob Agents Chemother* **2019,** *63* (8). DOI: 10.1128/AAC.00350-19.
65. Oganesyan, V.; Peng, L.; Damschroder, M. M.; Cheng, L.; Sadowska, A.; Tkaczyk, C.; Sellman, B. R.; Wu, H.; Dall'Acqua, W. F., Mechanisms of neutralization of a human anti-α-toxin antibody. *J Biol Chem* **2014,** *289* (43), 29874-80. DOI: 10.1074/jbc.M114.601328.
66. Hua, L.; Hilliard, J. J.; Shi, Y.; Tkaczyk, C.; Cheng, L. I.; Yu, X.; Datta, V.; Ren, S.; Feng, H.; Zinsou, R.; Keller, A.; O'Day, T.; Du, Q.; Cheng, L.; Damschroder, M.; Robbie, G.; Suzich, J.; Stover, C. K.; Sellman, B. R., Assessment of an anti-alpha-toxin monoclonal antibody for prevention and treatment of *Staphylococcus aureus*-induced pneumonia. *Antimicrob Agents Chemother* **2014,** *58* (2), 1108-17. DOI: 10.1128/AAC.02190-13.
67. Yu, X. Q.; Robbie, G. J.; Wu, Y.; Esser, M. T.; Jensen, K.; Schwartz, H. I.; Bellamy, T.; Hernandez-Illas, M.; Jafri, H. S., Safety, Tolerability, and Pharmacokinetics of MEDI4893, an Investigational, Extended-Half-Life, Anti-*Staphylococcus aureus* Alpha-Toxin Human Monoclonal Antibody, in Healthy Adults. *Antimicrob Agents Chemother* **2017,** *61* (1). DOI: 10.1128/AAC.01020-16.
68. François, B.; Mercier, E.; Gonzalez, C.; Asehnoune, K.; Nseir, S.; Fiancette, M.; Desachy, A.; Plantefève, G.; Meziani, F.; de Lame, P. A.; Laterre, P. F.; group, M. s., Safety and tolerability of a single administration of AR-301, a human monoclonal antibody, in ICU patients with severe pneumonia caused by *Staphylococcus aureus*: first-in-human trial. *Intensive Care Med* **2018,** *44* (11), 1787-1796. DOI: 10.1007/s00134-018-5229-2.
69. Tarr, P. I.; Gordon, C. A.; Chandler, W. L., Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **2005,** *365* (9464), 1073-86. DOI: 10.1016/S0140-6736(05)71144-2.
70. Kakoullis, L.; Papachristodoulou, E.; Chra, P.; Panos, G., Shiga toxin-induced haemolytic uraemic syndrome and the role of antibiotics: a global overview. *J Infect* **2019,** *79* (2), 75-94. DOI: 10.1016/j.jinf.2019.05.018.
71. Strockbine, N. A.; Marques, L. R.; Holmes, R. K.; O'Brien, A. D., Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. *Infect Immun* **1985,** *50* (3), 695-700. DOI: 10.1128/IAI.50.3.695-700.1985.

72. Melton-Celsa, A. R., Shiga Toxin (Stx) Classification, Structure, and Function. *Microbiol Spectr* **2014,** *2* (4), EHEC-0024-2013. DOI: 10.1128/microbiolspec.EHEC-0024-2013.
73. Melton-Celsa, A. R.; Carvalho, H. M.; Thuning-Roberson, C.; O'Brien, A. D., Protective efficacy and pharmacokinetics of human/mouse chimeric anti-Stx1 and anti-Stx2 antibodies in mice. *Clin Vaccine Immunol* **2015,** *22* (4), 448-55. DOI: 10.1128/CVI.00022-15.
74. Perera, L. P.; Marques, L. R.; O'Brien, A. D., Isolation and characterization of monoclonal antibodies to Shiga-like toxin II of enterohemorrhagic *Escherichia coli* and use of the monoclonal antibodies in a colony enzyme-linked immunosorbent assay. *J Clin Microbiol* **1988,** *26* (10), 2127-31. DOI: 10.1128/JCM.26.10.2127-2131.1988.
75. Smith, M. J.; Carvalho, H. M.; Melton-Celsa, A. R.; O'Brien, A. D., The 13C4 monoclonal antibody that neutralizes Shiga toxin Type 1 (Stx1) recognizes three regions on the Stx1 B subunit and prevents Stx1 from binding to its eukaryotic receptor globotriaosylceramide. *Infect Immun* **2006,** *74* (12), 6992-8. DOI: 10.1128/IAI.01247-06.
76. Smith, M. J.; Melton-Celsa, A. R.; Sinclair, J. F.; Carvalho, H. M.; Robinson, C. M.; O'Brien, A. D., Monoclonal antibody 11E10, which neutralizes shiga toxin type 2 (Stx2), recognizes three regions on the Stx2 A subunit, blocks the enzymatic action of the toxin *in vitro*, and alters the overall cellular distribution of the toxin. *Infect Immun* **2009,** *77* (7), 2730-40. DOI: 10.1128/IAI.00005-09.
77. Dowling, T. C.; Chavaillaz, P. A.; Young, D. G.; Melton-Celsa, A.; O'Brien, A.; Thuning-Roberson, C.; Edelman, R.; Tacket, C. O., Phase 1 safety and pharmacokinetic study of chimeric murine-human monoclonal antibody c alpha Stx2 administered intravenously to healthy adult volunteers. *Antimicrob Agents Chemother* **2005,** *49* (5), 1808-12. DOI: 10.1128/AAC.49.5.1808-1812.2005.
78. Bitzan, M.; Poole, R.; Mehran, M.; Sicard, E.; Brockus, C.; Thuning-Roberson, C.; Rivière, M., Safety and pharmacokinetics of chimeric anti-Shiga toxin 1 and anti-Shiga toxin 2 monoclonal antibodies in healthy volunteers. *Antimicrob Agents Chemother* **2009,** *53* (7), 3081-7. DOI: 10.1128/AAC.01661-08.
79. Melton-Celsa, A. R.; O'Brien, A. D., New Therapeutic Developments against Shiga Toxin-Producing *Escherichia coli*. *Microbiol Spectr* **2014,** *2* (5). DOI: 10.1128/microbiolspec.EHEC-0013-2013.
80. López, E. L.; Contrini, M. M.; Glatstein, E.; González Ayala, S.; Santoro, R.; Allende, D.; Ezcurra, G.; Teplitz, E.; Koyama, T.; Matsumoto, Y.; Sato, H.; Sakai, K.; Hoshide, S.; Komoriya, K.; Morita, T.; Harning, R.; Brookman, S., Safety and pharmacokinetics of urtoxazumab, a humanized monoclonal antibody, against Shiga-like toxin 2 in healthy adults and in pediatric patients infected with Shiga-like toxin-producing *Escherichia coli*. *Antimicrob Agents Chemother* **2010,** *54* (1), 239-43. DOI: 10.1128/AAC.00343-09.
81. Nakao, H.; Kiyokawa, N.; Fujimoto, J.; Yamasaki, S.; Takeda, T., Monoclonal antibody to Shiga toxin 2 which blocks receptor binding and neutralizes cytotoxicity. *Infect Immun* **1999,** *67* (11), 5717-22. DOI: 10.1128/IAI.67.11.5717-5722.1999.
82. Kimura, T.; Co, M. S.; Vasquez, M.; Wei, S.; Xu, H.; Tani, S.; Sakai, Y.; Kawamura, T.; Matsumoto, Y.; Nakao, H.; Takeda, T., Development of humanized monoclonal antibody TMA-15 which neutralizes Shiga toxin 2. *Hybrid Hybridomics* **2002,** *21* (3), 161-8. DOI: 10.1089/153685902760173872.
83. Yamagami, S.; Motoki, M.; Kimura, T.; Izumi, H.; Takeda, T.; Katsuura, Y.; Matsumoto, Y., Efficacy of postinfection treatment with anti-Shiga toxin (Stx) 2 humanized monoclonal antibody TMA-15 in mice lethally challenged with Stx-producing *Escherichia coli*. *J Infect Dis* **2001,** *184* (6), 738-42. DOI: 10.1086/323082.
84. Moxley, R. A.; Francis, D. H.; Tamura, M.; Marx, D. B.; Santiago-Mateo, K.; Zhao, M., Efficacy of Urtoxazumab (TMA-15 Humanized Monoclonal Antibody Specific for Shiga Toxin 2) Against Post-Diarrheal Neurological Sequelae Caused by *Escherichia coli* O157:H7 Infection in the Neonatal Gnotobiotic Piglet Model. *Toxins (Basel)* **2017,** *9* (2). DOI: 10.3390/toxins9020049.
85. Kilgore, P. E.; Salim, A. M.; Zervos, M. J.; Schmitt, H. J., Pertussis: Microbiology, Disease, Treatment, and Prevention. *Clin Microbiol Rev* **2016,** *29* (3), 449-86. DOI: 10.1128/CMR.00083-15.
86. Skoff, T. H.; Baumbach, J.; Cieslak, P. R., Tracking Pertussis and Evaluating Control Measures through Enhanced Pertussis Surveillance, Emerging Infections Program, United States. *Emerg Infect Dis* **2015,** *21* (9), 1568-73. DOI: 10.3201/eid2109.150023.
87. Guillot, S.; Descours, G.; Gillet, Y.; Etienne, J.; Floret, D.; Guiso, N., Macrolide-resistant *Bordetella pertussis* infection in newborn girl, France. *Emerg Infect Dis* **2012,** *18* (6), 966-8. DOI: 10.3201/eid1806.120091.

88. Wang, Z.; Li, Y.; Hou, T.; Liu, X.; Liu, Y.; Yu, T.; Chen, Z.; Gao, Y.; Li, H.; He, Q., Appearance of macrolide-resistant *Bordetella pertussis* strains in China. *Antimicrob Agents Chemother* **2013,** *57* (10), 5193-4. DOI: 10.1128/AAC.01081-13.
89. Nguyen, A. W.; DiVenere, A. M.; Papin, J. F.; Connelly, S.; Kaleko, M.; Maynard, J. A., Neutralization of pertussis toxin by a single antibody prevents clinical pertussis in neonatal baboons. *Sci Adv* **2020,** *6* (6), eaay9258. DOI: 10.1126/sciadv.aay9258.
90. Acquaye-Seedah, E.; Huang, Y.; Sutherland, J. N.; DiVenere, A. M.; Maynard, J. A., Humanised monoclonal antibodies neutralise pertussis toxin by receptor blockade and reduced retrograde trafficking. *Cell Microbiol* **2018,** *20* (12), e12948. DOI: 10.1111/cmi.12948.
91. Nguyen, A. W.; Wagner, E. K.; Laber, J. R.; Goodfield, L. L.; Smallridge, W. E.; Harvill, E. T.; Papin, J. F.; Wolf, R. F.; Padlan, E. A.; Bristol, A.; Kaleko, M.; Maynard, J. A., A cocktail of humanized anti-pertussis toxin antibodies limits disease in murine and baboon models of whooping cough. *Sci Transl Med* **2015,** *7* (316), 316ra195. DOI: 10.1126/scitranslmed.aad0966.
92. Wagner, E. K.; Wang, X.; Bui, A.; Maynard, J. A., Synergistic Neutralization of Pertussis Toxin by a Bispecific Antibody In Vitro and In Vivo. *Clin Vaccine Immunol* **2016,** *23* (11), 851-862. DOI: 10.1128/CVI.00371-16.
93. Sato, H.; Sato, Y., Protective activities in mice of monoclonal antibodies against pertussis toxin. *Infect Immun* **1990,** *58* (10), 3369-74. DOI: 10.1128/IAI.58.10.3369-3374.1990.
94. Bates, A.; Power, C. A., David vs. Goliath: The Structure, Function, and Clinical Prospects of Antibody Fragments. *Antibodies (Basel)* **2019,** *8* (2). DOI: 10.3390/antib8020028.
95. Chen, G.; Karauzum, H.; Long, H.; Carranza, D.; Holtsberg, F. W.; Howell, K. A.; Abaandou, L.; Zhang, B.; Jarvik, N.; Ye, W.; Liao, G. C.; Gross, M. L.; Leung, D. W.; Amarasinghe, G. K.; Aman, M. J.; Sidhu, S. S., Potent Neutralization of Staphylococcal Enterotoxin B *In Vivo* by Antibodies that Block Binding to the T-Cell Receptor. *J Mol Biol* **2019,** *431* (21), 4354-4367. DOI: 10.1016/j.jmb.2019.03.017.
96. Fühner, V.; Heine, P. A.; Helmsing, S.; Goy, S.; Heidepriem, J.; Loeffler, F. F.; Dübel, S.; Gerhard, R.; Hust, M., Development of Neutralizing and Non-neutralizing Antibodies Targeting Known and Novel Epitopes of TcdB of *Clostridioides difficile*. *Front Microbiol* **2018,** *9*, 2908. DOI: 10.3389/fmicb.2018.02908.
97. Godakova, S. A.; Noskov, A. N.; Vinogradova, I. D.; Ugriumova, G. A.; Solovyev, A. I.; Esmagambetov, I. B.; Tukhvatulin, A. I.; Logunov, D. Y.; Naroditsky, B. S.; Shcheblyakov, D. V.; Gintsburg, A. L., Camelid VHHs Fused to Human Fc Fragments Provide Long Term Protection Against Botulinum Neurotoxin A in Mice. *Toxins (Basel)* **2019,** *11* (8). DOI: 10.3390/toxins11080464.
98. Lam, K. H.; Tremblay, J. M.; Vazquez-Cintron, E.; Perry, K.; Ondeck, C.; Webb, R. P.; McNutt, P. M.; Shoemaker, C. B.; Jin, R., Structural Insights into Rational Design of Single-Domain Antibody-Based Antitoxins against Botulinum Neurotoxins. *Cell Rep* **2020,** *30* (8), 2526-2539.e6. DOI: 10.1016/j.celrep.2020.01.107.
99. Simeon, R.; Chen, Z., *In vitro*-engineered non-antibody protein therapeutics. *Protein Cell* **2018,** *9* (1), 3-14. DOI: 10.1007/s13238-017-0386-6.
100. Jacobs, S. A.; Gibbs, A. C.; Conk, M.; Yi, F.; Maguire, D.; Kane, C.; O'Neil, K. T., Fusion to a highly stable consensus albumin binding domain allows for tunable pharmacokinetics. *Protein Eng Des Sel* **2015,** *28* (10), 385-93. DOI: 10.1093/protein/gzv040.
101. Simeon, R.; Jiang, M.; Chamoun-Emanuelli, A. M.; Yu, H.; Zhang, Y.; Meng, R.; Peng, Z.; Jakana, J.; Zhang, J.; Feng, H.; Chen, Z., Selection and characterization of ultrahigh potency designed ankyrin repeat protein inhibitors of *C. difficile* toxin B. *PLoS Biol* **2019,** *17* (6), e3000311. DOI: 10.1371/journal.pbio.3000311.
102. Peng, Z.; Simeon, R.; Mitchell, S. B.; Zhang, J.; Feng, H.; Chen, Z., Designed Ankyrin Repeat Protein (DARPin) Neutralizers of TcdB from *Clostridium difficile* Ribotype 027. *mSphere* **2019,** *4* (5). DOI: 10.1128/mSphere.00596-19.
103. Caputi, A. P.; Navarra, P., Beyond antibodies: ankyrins and DARPins. From basic research to drug approval. *Curr Opin Pharmacol* **2020,** *51*, 93-101. DOI: 10.1016/j.coph.2020.05.004.
104. Chan, R.; Buckley, P. T.; O'Malley, A.; Sause, W. E.; Alonzo, F.; Lubkin, A.; Boguslawski, K. M.; Payne, A.; Fernandez, J.; Strohl, W. R.; Whitaker, B.; Lynch, A. S.; Torres, V. J., Identification of biologic agents to neutralize the bicomponent leukocidins of *Staphylococcus aureus*. *Sci Transl Med* **2019,** *11* (475). DOI: 10.1126/scitranslmed.aat0882.

105. Diem, M. D.; Hyun, L.; Yi, F.; Hippensteel, R.; Kuhar, E.; Lowenstein, C.; Swift, E. J.; O'Neil, K. T.; Jacobs, S. A., Selection of high-affinity Centyrin FN3 domains from a simple library diversified at a combination of strand and loop positions. *Protein Eng Des Sel* **2014,** *27* (10), 419-29. DOI: 10.1093/protein/gzu016.
106. Jacobs, S. A.; Diem, M. D.; Luo, J.; Teplyakov, A.; Obmolova, G.; Malia, T.; Gilliland, G. L.; O'Neil, K. T., Design of novel FN3 domains with high stability by a consensus sequence approach. *Protein Eng Des Sel* **2012,** *25* (3), 107-17. DOI: 10.1093/protein/gzr064.
107. Armstrong, G. D.; Rowe, P. C.; Goodyer, P.; Orrbine, E.; Klassen, T. P.; Wells, G.; MacKenzie, A.; Lior, H.; Blanchard, C.; Auclair, F., A phase I study of chemically synthesized verotoxin (Shiga-like toxin) Pk-trisaccharide receptors attached to chromosorb for preventing hemolytic-uremic syndrome. *J Infect Dis* **1995,** *171* (4), 1042-5. DOI: 10.1093/infdis/171.4.1042.
108. Trachtman, H.; Cnaan, A.; Christen, E.; Gibbs, K.; Zhao, S.; Acheson, D. W.; Weiss, R.; Kaskel, F. J.; Spitzer, A.; Hirschman, G. H.; Trial, I. o. t. H.-S. P. M. C., Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: a randomized controlled trial. *JAMA* **2003,** *290* (10), 1337-44. DOI: 10.1001/jama.290.10.1337.
109. Kurtz, C. B.; Cannon, E. P.; Brezzani, A.; Pitruzzello, M.; Dinardo, C.; Rinard, E.; Acheson, D. W.; Fitzpatrick, R.; Kelly, P.; Shackett, K.; Papoulis, A. T.; Goddard, P. J.; Barker, R. H.; Palace, G. P.; Klinger, J. D., GT160-246, a toxin binding polymer for treatment of *Clostridium difficile* colitis. *Antimicrob Agents Chemother* **2001,** *45* (8), 2340-7. DOI: 10.1128/AAC.45.8.2340-2347.2001.
110. Braunlin, W.; Xu, Q.; Hook, P.; Fitzpatrick, R.; Klinger, J. D.; Burrier, R.; Kurtz, C. B., Toxin binding of tolevamer, a polyanionic drug that protects against antibiotic-associated diarrhea. *Biophys J* **2004,** *87* (1), 534-9. DOI: 10.1529/biophysj.104.041277.
111. Hinkson, P. L.; Dinardo, C.; DeCiero, D.; Klinger, J. D.; Barker, R. H., Tolevamer, an anionic polymer, neutralizes toxins produced by the BI/027 strains of *Clostridium difficile*. *Antimicrob Agents Chemother* **2008,** *52* (6), 2190-5. DOI: 10.1128/AAC.00041-08.
112. Louie, T. J.; Peppe, J.; Watt, C. K.; Johnson, D.; Mohammed, R.; Dow, G.; Weiss, K.; Simon, S.; John, J. F.; Garber, G.; Chasan-Taber, S.; Davidson, D. M.; Group, T. S. I., Tolevamer, a novel nonantibiotic polymer, compared with vancomycin in the treatment of mild to moderately severe *Clostridium difficile*-associated diarrhea. *Clin Infect Dis* **2006,** *43* (4), 411-20. DOI: 10.1086/506349.
113. Peppe, J.; Porzio, A.; Davidson, D. M., A new formulation of tolevamer, a novel nonantibiotic polymer, is safe and well-tolerated in healthy volunteers: a randomized phase I trial. *Br J Clin Pharmacol* **2008,** *66* (1), 102-9. DOI: 10.1111/j.1365-2125.2008.03151.x.
114. Johnson, S.; Louie, T. J.; Gerding, D. N.; Cornely, O. A.; Chasan-Taber, S.; Fitts, D.; Gelone, S. P.; Broom, C.; Davidson, D. M.; investigators, P. A. f. C. T. P., Vancomycin, metronidazole, or tolevamer for *Clostridium difficile* infection: results from two multinational, randomized, controlled trials. *Clin Infect Dis* **2014,** *59* (3), 345-54. DOI: 10.1093/cid/ciu313.
115. Mulvey, G. L.; Marcato, P.; Kitov, P. I.; Sadowska, J.; Bundle, D. R.; Armstrong, G. D., Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. *J Infect Dis* **2003,** *187* (4), 640-9. DOI: 10.1086/373996.
116. Kitov, P. I.; Sadowska, J. M.; Mulvey, G.; Armstrong, G. D.; Ling, H.; Pannu, N. S.; Read, R. J.; Bundle, D. R., Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature* **2000,** *403* (6770), 669-72. DOI: 10.1038/35001095.
117. Nishikawa, K.; Matsuoka, K.; Watanabe, M.; Igai, K.; Hino, K.; Hatano, K.; Yamada, A.; Abe, N.; Terunuma, D.; Kuzuhara, H.; Natori, Y., Identification of the optimal structure required for a Shiga toxin neutralizer with oriented carbohydrates to function in the circulation. *J Infect Dis* **2005,** *191* (12), 2097-105. DOI: 10.1086/430388.
118. Nishikawa, K.; Matsuoka, K.; Kita, E.; Okabe, N.; Mizuguchi, M.; Hino, K.; Miyazawa, S.; Yamasaki, C.; Aoki, J.; Takashima, S.; Yamakawa, Y.; Nishijima, M.; Terunuma, D.; Kuzuhara, H.; Natori, Y., A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin-producing *Escherichia coli* O157:H7. *Proc Natl Acad Sci U S A* **2002,** *99* (11), 7669-74. DOI: 10.1073/pnas.112058999.
119. Paton, A. W.; Morona, R.; Paton, J. C., A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. *Nat Med* **2000,** *6* (3), 265-70. DOI: 10.1038/73111.
120. Paton, A. W.; Chen, A. Y.; Wang, H.; McAllister, L. J.; Höggerl, F.; Mayr, U. B.; Shewell, L. K.; Jennings, M. P.; Morona, R.; Lubitz, W.; Paton, J. C., Protection against Shiga-Toxigenic *Escherichia*

coli by Non-Genetically Modified Organism Receptor Mimic Bacterial Ghosts. *Infect Immun* **2015,** *83* (9), 3526-33. DOI: 10.1128/IAI.00669-15.

121. Subramanian, K.; Iovino, F.; Tsikourkitoudi, V.; Merkl, P.; Ahmed, S.; Berry, S. B.; Aschtgen, M. S.; Svensson, M.; Bergman, P.; Sotiriou, G. A.; Henriques-Normark, B., Mannose receptor-derived peptides neutralize pore-forming toxins and reduce inflammation and development of pneumococcal disease. *EMBO Mol Med* **2020,** *12* (11), e12695. DOI: 10.15252/emmm.202012695.
122. Subramanian, K.; Neill, D. R.; Malak, H. A.; Spelmink, L.; Khandaker, S.; Dalla Libera Marchiori, G.; Dearing, E.; Kirby, A.; Yang, M.; Achour, A.; Nilvebrant, J.; Nygren, P.; Plant, L.; Kadioglu, A.; Henriques-Normark, B., Pneumolysin binds to the mannose receptor C type 1 (MRC-1) leading to anti-inflammatory responses and enhanced pneumococcal survival. *Nat Microbiol* **2019,** *4* (1), 62-70. DOI: 10.1038/s41564-018-0280-x.
123. Henry, B. D.; Neill, D. R.; Becker, K. A.; Gore, S.; Bricio-Moreno, L.; Ziobro, R.; Edwards, M. J.; Mühlemann, K.; Steinmann, J.; Kleuser, B.; Japtok, L.; Luginbühl, M.; Wolfmeier, H.; Scherag, A.; Gulbins, E.; Kadioglu, A.; Draeger, A.; Babiychuk, E. B., Engineered liposomes sequester bacterial exotoxins and protect from severe invasive infections in mice. *Nat Biotechnol* **2015,** *33* (1), 81-8. DOI: 10.1038/nbt.3037.
124. Keller, M. D.; Ching, K. L.; Liang, F. X.; Dhabaria, A.; Tam, K.; Ueberheide, B. M.; Unutmaz, D.; Torres, V. J.; Cadwell, K., Decoy exosomes provide protection against bacterial toxins. *Nature* **2020,** *579* (7798), 260-264. DOI: 10.1038/s41586-020-2066-6.
125. Coburn, P. S.; Miller, F. C.; LaGrow, A. L.; Land, C.; Mursalin, H.; Livingston, E.; Amayem, O.; Chen, Y.; Gao, W.; Zhang, L.; Callegan, M. C., Disarming Pore-Forming Toxins with Biomimetic Nanosponges in Intraocular Infections. *mSphere* **2019,** *4* (3). DOI: 10.1128/mSphere.00262-19.
126. Chen, Y.; Chen, M.; Zhang, Y.; Lee, J. H.; Escajadillo, T.; Gong, H.; Fang, R. H.; Gao, W.; Nizet, V.; Zhang, L., Broad-Spectrum Neutralization of Pore-Forming Toxins with Human Erythrocyte Membrane-Coated Nanosponges. *Adv Healthc Mater* **2018,** *7* (13), e1701366. DOI: 10.1002/adhm.201701366.
127. Wu, X.; Li, Y.; Raza, F.; Wang, X.; Zhang, S.; Rong, R.; Qiu, M.; Su, J., Red Blood Cell Membrane-Camouflaged Tedizolid Phosphate-Loaded PLGA Nanoparticles for Bacterial-Infection Therapy. *Pharmaceutics* **2021,** *13* (1). DOI: 10.3390/pharmaceutics13010099.
128. Jiang, L.; Zhu, Y.; Luan, P.; Xu, J.; Ru, G.; Fu, J. G.; Sang, N.; Xiong, Y.; He, Y.; Lin, G. Q.; Wang, J.; Zhang, J.; Li, R., Bacteria-anchoring hybrid liposome capable of absorbing multiple toxins for anivirulence therapy of *Escherichia coli* infection. *ACS Nano* **2021,** *15* (3), 4173-4185. DOI: 10.1021/acsnano.0c04800.
129. Lin, A.; Liu, Y.; Zhu, X.; Chen, X.; Liu, J.; Zhou, Y.; Qin, X., Bacteria-Responsive Biomimetic Selenium Nanosystem for Multidrug-Resistant Bacterial Infection Detection and Inhibition. *ACS Nano* **2019,** *13* (12), 13965-13984. DOI: 10.1021/acsnano.9b05766.
130. Huang, N. J.; Pishesha, N.; Mukherjee, J.; Zhang, S.; Deshycka, R.; Sudaryo, V.; Dong, M.; Shoemaker, C. B.; Lodish, H. F., Genetically engineered red cells expressing single domain camelid antibodies confer long-term protection against botulinum neurotoxin. *Nat Commun* **2017,** *8* (1), 423. DOI: 10.1038/s41467-017-00448-0.
131. Laterre, P. F.; Colin, G.; Dequin, P. F.; Dugernier, T.; Boulain, T.; Azeredo da Silveira, S.; Lajaunias, F.; Perez, A.; François, B., CAL02, a novel antitoxin liposomal agent, in severe pneumococcal pneumonia: a first-in-human, double-blind, placebo-controlled, randomised trial. *Lancet Infect Dis* **2019,** *19* (6), 620-630. DOI: 10.1016/S1473-3099(18)30805-3.
132. Song, L.; Hobaugh, M. R.; Shustak, C.; Cheley, S.; Bayley, H.; Gouaux, J. E., Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **1996,** *274* (5294), 1859-66. DOI: 10.1126/science.274.5294.1859.
133. Reyes-Robles, T.; Lubkin, A.; Alonzo, F.; Lacy, D. B.; Torres, V. J., Exploiting dominant-negative toxins to combat *Staphylococcus aureus* pathogenesis. *EMBO Rep* **2016,** *17* (3), 428-40. DOI: 10.15252/embr.201540994.
134. Pelish, T. M.; McClain, M. S., Dominant-negative inhibitors of the *Clostridium perfringens* epsilon-toxin. *J Biol Chem* **2009,** *284* (43), 29446-53. DOI: 10.1074/jbc.M109.021782.
135. Ivie, S. E.; McClain, M. S.; Torres, V. J.; Algood, H. M.; Lacy, D. B.; Yang, R.; Blanke, S. R.; Cover, T. L., *Helicobacter pylori* VacA subdomain required for intracellular toxin activity and assembly of functional oligomeric complexes. *Infect Immun* **2008,** *76* (7), 2843-51. DOI: 10.1128/IAI.01664-07.

136. Cao, S.; Guo, A.; Liu, Z.; Tan, Y.; Wu, G.; Zhang, C.; Zhao, Y.; Chen, H., Investigation of new dominant-negative inhibitors of anthrax protective antigen mutants for use in therapy and vaccination. *Infect Immun* **2009,** *77* (10), 4679-87. DOI: 10.1128/IAI.00264-09.
137. Sellman, B. R.; Mourez, M.; Collier, R. J., Dominant-negative mutants of a toxin subunit: an approach to therapy of anthrax. *Science* **2001,** *292* (5517), 695-7. DOI: 10.1126/science.109563.
138. Singh, Y.; Khanna, H.; Chopra, A. P.; Mehra, V., A dominant negative mutant of *Bacillus anthracis* protective antigen inhibits anthrax toxin action in vivo. *J Biol Chem* **2001,** *276* (25), 22090-4. DOI: 10.1074/jbc.M010222200.
139. Larabee, J. L.; Bland, S. J.; Hunt, J. J.; Ballard, J. D., Intrinsic Toxin-Derived Peptides Destabilize and Inactivate *Clostridium difficile* TcdB. *mBio* **2017,** *8* (3). DOI: 10.1128/mBio.00503-17.
140. Markossian, S.; Ang, K. K.; Wilson, C. G.; Arkin, M. R., Small-Molecule Screening for Genetic Diseases. *Annu Rev Genomics Hum Genet* **2018,** *19*, 263-288. DOI: 10.1146/annurev-genom-083117-021452.
141. Tam, J.; Beilhartz, G. L.; Auger, A.; Gupta, P.; Therien, A. G.; Melnyk, R. A., Small molecule inhibitors of *Clostridium difficile* toxin B-induced cellular damage. *Chem Biol* **2015,** *22* (2), 175-85. DOI: 10.1016/j.chembiol.2014.12.010.
142. Liu, J.; Kozhaya, L.; Torres, V. J.; Unutmaz, D.; Lu, M., Structure-based discovery of a small-molecule inhibitor of methicillin-resistant. *J Biol Chem* **2020,** *295* (18), 5944-5959. DOI: 10.1074/jbc.RA120.012697.
143. Dorlo, T. P.; Balasegaram, M.; Beijnen, J. H.; de Vries, P. J., Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J Antimicrob Chemother* **2012,** *67* (11), 2576-97. DOI: 10.1093/jac/dks275.
144. Marozsan, A. J.; Ma, D.; Nagashima, K. A.; Kennedy, B. J.; Kang, Y. K.; Arrigale, R. R.; Donovan, G. P.; Magargal, W. W.; Maddon, P. J.; Olson, W. C., Protection against *Clostridium difficile* infection with broadly neutralizing antitoxin monoclonal antibodies. *J Infect Dis* **2012,** *206* (5), 706-13. DOI: 10.1093/infdis/jis416.
145. Kroh, H. K.; Chandrasekaran, R.; Rosenthal, K.; Woods, R.; Jin, X.; Ohi, M. D.; Nyborg, A. C.; Rainey, G. J.; Warrener, P.; Spiller, B. W.; Lacy, D. B., Use of a neutralizing antibody helps identify structural features critical for binding of *Clostridium difficile* TcdA to the host cell surface. *J Biol Chem* **2017,** *292* (35), 14401-14412. DOI: 10.1074/jbc.M117.781112.
146. Kroh, H. K.; Chandrasekaran, R.; Zhang, Z.; Rosenthal, K.; Woods, R.; Jin, X.; Nyborg, A. C.; Rainey, G. J.; Warrener, P.; Melnyk, R. A.; Spiller, B. W.; Lacy, D. B., A neutralizing antibody that blocks delivery of the enzymatic cargo of *Clostridium difficile* TcdB into host cells. *J Biol Chem* **2018,** *293* (3), 941-952. DOI: 10.1074/jbc.M117.813428.
147. Mechaly, A.; Levy, H.; Epstein, E.; Rosenfeld, R.; Marcus, H.; Ben-Arie, E.; Shafferman, A.; Ordentlich, A.; Mazor, O., A novel mechanism for antibody-based anthrax toxin neutralization: inhibition of prepore-to-pore conversion. *J Biol Chem* **2012,** *287* (39), 32665-73. DOI: 10.1074/jbc.M112.400473.
148. Krautz-Peterson, G.; Chapman-Bonofiglio, S.; Boisvert, K.; Feng, H.; Herman, I. M.; Tzipori, S.; Sheoran, A. S., Intracellular neutralization of shiga toxin 2 by an a subunit-specific human monoclonal antibody. *Infect Immun* **2008,** *76* (5), 1931-9. DOI: 10.1128/IAI.01282-07.
149. Azad, G. K.; Tomar, R. S., Ebselen, a promising antioxidant drug: mechanisms of action and targets of biological pathways. *Mol Biol Rep* **2014,** *41* (8), 4865-79. DOI: 10.1007/s11033-014-3417-x.
150. Bender, K. O.; Garland, M.; Ferreyra, J. A.; Hryckowian, A. J.; Child, M. A.; Puri, A. W.; Solow-Cordero, D. E.; Higginbottom, S. K.; Segal, E.; Banaei, N.; Shen, A.; Sonnenburg, J. L.; Bogyo, M., A small-molecule antivirulence agent for treating *Clostridium difficile* infection. *Sci Transl Med* **2015,** *7* (306), 306ra148. DOI: 10.1126/scitranslmed.aac9103.
151. Bender, K. O.; Garland, M.; Bogyo, M., Response to Comment on "A small-molecule antivirulence agent for treating *Clostridium difficile* infection". *Sci Transl Med* **2016,** *8* (370), 370tr2. DOI: 10.1126/scitranslmed.aaf3410.
152. Beilhartz, G. L.; Tam, J.; Zhang, Z.; Melnyk, R. A., Comment on "A small-molecule antivirulence agent for treating *Clostridium difficile* infection". *Sci Transl Med* **2016,** *8* (370), 370tc2. DOI: 10.1126/scitranslmed.aad8926.
153. Garland, M.; Hryckowian, A. J.; Tholen, M.; Bender, K. O.; Van Treuren, W. W.; Loscher, S.; Sonnenburg, J. L.; Bogyo, M., The Clinical Drug Ebselen Attenuates Inflammation and Promotes

Microbiome Recovery in Mice after Antibiotic Treatment for CDI. *Cell Rep Med* **2020,** *1* (1). DOI: 10.1016/j.xcrm.2020.100005.
154. Shen, A.; Lupardus, P. J.; Albrow, V. E.; Guzzetta, A.; Powers, J. C.; Garcia, K. C.; Bogyo, M., Mechanistic and structural insights into the proteolytic activation of *Vibrio cholerae* MARTX toxin. *Nat Chem Biol* **2009,** *5* (7), 469-78. DOI: 10.1038/nchembio.178.
155. Puri, A. W.; Lupardus, P. J.; Deu, E.; Albrow, V. E.; Garcia, K. C.; Bogyo, M.; Shen, A., Rational design of inhibitors and activity-based probes targeting *Clostridium difficile* virulence factor TcdB. *Chem Biol* **2010,** *17* (11), 1201-11. DOI: 10.1016/j.chembiol.2010.09.011.
156. Ivarsson, M. E.; Durantie, E.; Huberli, C.; Huwiler, S.; Hegde, C.; Friedman, J.; Altamura, F.; Lu, J.; Verdu, E. F.; Bercik, P.; Logan, S. M.; Chen, W.; Leroux, J. C.; Castagner, B., Small-Molecule Allosteric Triggers of *Clostridium difficile* Toxin B Auto-proteolysis as a Therapeutic Strategy. *Cell Chem Biol* **2019,** *26* (1), 17-26.e13. DOI: 10.1016/j.chembiol.2018.10.002.
157. Madden, J. C.; Ruiz, N.; Caparon, M., Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. *Cell* **2001,** *104* (1), 143-52.
158. Rogel, A.; Hanski, E., Distinct steps in the penetration of adenylate cyclase toxin of Bordetella pertussis into sheep erythrocytes. Translocation of the toxin across the membrane. *J Biol Chem* **1992,** *267* (31), 22599-605.
159. Letourneau, J. J.; Stroke, I. L.; Hilbert, D. W.; Sturzenbecker, L. J.; Marinelli, B. A.; Quintero, J. G.; Sabalski, J.; Ma, L.; Diller, D. J.; Stein, P. D.; Webb, M. L., Identification and initial optimization of inhibitors of *Clostridium difficile* (*C. difficile*) toxin B (TcdB). *Bioorg Med Chem Lett* **2018,** *28* (4), 756-761. DOI: 10.1016/j.bmcl.2018.01.005.
160. Letourneau, J. J.; Stroke, I. L.; Hilbert, D. W.; Cole, A. G.; Sturzenbecker, L. J.; Marinelli, B. A.; Quintero, J. G.; Sabalski, J.; Li, Y.; Ma, L.; Pechik, I.; Stein, P. D.; Webb, M. L., Synthesis and SAR studies of novel benzodiazepinedione-based inhibitors of *Clostridium difficile* (*C. difficile*) toxin B (TcdB). *Bioorg Med Chem Lett* **2018,** *28* (23-24), 3601-3605. DOI: 10.1016/j.bmcl.2018.10.047.
161. Stroke, I. L.; Letourneau, J. J.; Miller, T. E.; Xu, Y.; Pechik, I.; Savoly, D. R.; Ma, L.; Sturzenbecker, L. J.; Sabalski, J.; Stein, P. D.; Webb, M. L.; Hilbert, D. W., Treatment of *Clostridium difficile* Infection with a Small-Molecule Inhibitor of Toxin UDP-Glucose Hydrolysis Activity. *Antimicrob Agents Chemother* **2018,** *62* (5). DOI: 10.1128/AAC.00107-18.
162. Ashok, Y.; Miettinen, M.; Oliveira, D. K. H.; Tamirat, M. Z.; Näreoja, K.; Tiwari, A.; Hottiger, M. O.; Johnson, M. S.; Lehtiö, L.; Pulliainen, A. T., Discovery of Compounds Inhibiting the ADP-Ribosyltransferase Activity of Pertussis Toxin. *ACS Infect Dis* **2020,** *6* (4), 588-602. DOI: 10.1021/acsinfecdis.9b00412.
163. Turgeon, Z.; Jørgensen, R.; Visschedyk, D.; Edwards, P. R.; Legree, S.; McGregor, C.; Fieldhouse, R. J.; Mangroo, D.; Schapira, M.; Merrill, A. R., Newly discovered and characterized antivirulence compounds inhibit bacterial mono-ADP-ribosyltransferase toxins. *Antimicrob Agents Chemother* **2011,** *55* (3), 983-91. DOI: 10.1128/AAC.01164-10.
164. Jørgensen, R.; Purdy, A. E.; Fieldhouse, R. J.; Kimber, M. S.; Bartlett, D. H.; Merrill, A. R., Cholix toxin, a novel ADP-ribosylating factor from *Vibrio cholerae*. *J Biol Chem* **2008,** *283* (16), 10671-8. DOI: 10.1074/jbc.M710008200.
165. Maurer, B.; Mathias, U.; Papatheodorou, P.; Shekfeh, S.; Orth, J.; Jank, T.; Schwan, C.; Sippl, W.; Aktories, K.; Jung, M., From cosubstrate similarity to inhibitor diversity--inhibitors of ADP-ribosyltransferases from kinase inhibitor screening. *Mol Biosyst* **2011,** *7* (3), 799-808. DOI: 10.1039/c0mb00151a.
166. Zhang, G., Design, synthesis, and evaluation of bisubstrate analog inhibitors of cholera toxin. *Bioorg Med Chem Lett* **2008,** *18* (13), 3724-7. DOI: 10.1016/j.bmcl.2008.05.049.
167. Lugo, M. R.; Merrill, A. R., A comparative structure-function analysis of active-site inhibitors of *Vibrio cholerae* cholix toxin. *J Mol Recognit* **2015,** *28* (9), 539-52. DOI: 10.1002/jmr.2469.
168. Miettinen, M.; Vedantham, M.; Pulliainen, A. T., Host poly(ADP-ribose) polymerases (PARPs) in acute and chronic bacterial infections. *Microbes Infect* **2019,** *21* (10), 423-431. DOI: 10.1016/j.micinf.2019.06.002.
169. Nagy, C. F.; Leach, T. S.; Hoffman, J. H.; Czech, A.; Carpenter, S. E.; Guttendorf, R., Pharmacokinetics and Tolerability of Obiltoxaximab: A Report of 5 Healthy Volunteer Studies. *Clin Ther* **2016,** *38* (9), 2083-2097.e7. DOI: 10.1016/j.clinthera.2016.07.170.

170. Nayak, S. U.; Griffiss, J. M.; McKenzie, R.; Fuchs, E. J.; Jurao, R. A.; An, A. T.; Ahene, A.; Tomic, M.; Hendrix, C. W.; Zenilman, J. M., Safety and pharmacokinetics of XOMA 3AB, a novel mixture of three monoclonal antibodies against botulinum toxin A. *Antimicrob Agents Chemother* **2014,** *58* (9), 5047-53. DOI: 10.1128/AAC.02830-14.
171. Snow, D. M.; Riling, K.; Kimbler, A.; Espinoza, Y.; Wong, D.; Pham, K.; Martinez, Z.; Kraus, C. N.; Conrad, F.; Garcia-Rodriguez, C.; Cobb, R. R.; Marks, J. D.; Tomic, M. T., Safety and Pharmacokinetics of a Four Monoclonal Antibody Combination Against Botulinum C and D Neurotoxins. *Antimicrob Agents Chemother* **2019**. DOI: 10.1128/AAC.01270-19.