CHEMICAL REVIEWS

Review

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Chemical Strategies To Target Bacterial Virulence

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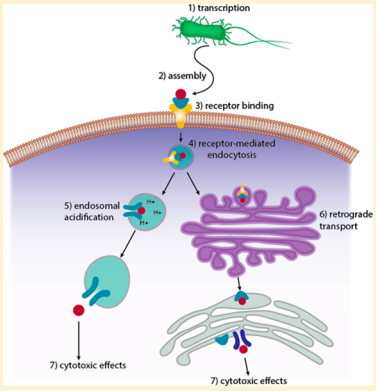
ABSTRACT: Antibiotic resistance is a significant emerging health threat. Exacerbating this problem is the overprescription of antibiotics as well as a lack of development of new antibacterial agents. A paradigm shift toward the development of nonantibiotic agents that target the virulence factors of bacterial pathogens is one way to begin to address the issue of resistance. Of particular interest are compounds targeting bacterial AB toxins that have the potential to protect against toxin-induced pathology without harming healthy commensal microbial flora. Development of successful antitoxin agents would likely decrease the use of antibiotics, thereby reducing selective pressure that leads to antibiotic resistance mutations. In addition, antitoxin agents are not only promising for therapeutic applications, but also can be used as tools for the continued study of bacterial pathogenesis. In this review, we discuss the growing number of examples of chemical entities designed to target exotoxin virulence factors from important human bacterial pathogens.



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1. INTRODUCTION

Antibiotic resistance is a growing problem that now causes 700 000 deaths per year worldwide.[1,2] In the 1930s, the advent of highly effective antibiotics rendered previously fatal bacterial infections minor inconveniences. Bayer's development of sulfa drugs[3] and Alexander Fleming's discovery of penicillin from the fungus *Penicillium chrysogenum*[4] spurred the development of additional classes of antimicrobials that have added to our arsenal of therapies to keep infectious diseases in check.[5] At the same time, however, once-susceptible microbes have gained resistance to the antibiotics that used to be viable treatment options. Today, antibiotic resistance is a serious and complex global health issue that threatens to undermine the significant scientific and medical progress made over the past 80 years to combat infectious diseases. The increase in antibiotic resistance is a multifactorial problem stemming from evolution by the bacterium, overuse and misuse of antibiotics, as well as a decrease in production of new antimicrobial agents with novel mechanisms of action. In 2013, the Centers for Disease Control and Prevention (CDC) recognized 17 bacteria (and one fungus) as threats to society, stratifying them based on their antibiotic resistance and severity of the threat (Table 1).[6]

The growing problem of bacterial resistance can be exemplified by the recent reports of "superbugs" in the mainstream media. The first known case of *Escherichia coli* harboring the *mcr-1* plasmid containing resistance genes for the antibiotic colistin was recently cultured from a woman with a urinary tract infection (UTI) in Pennsylvania.[7] The *mcr-1* plasmid was first reported in livestock in China, and has since spread across many continents.[7−9] Colistin is an antibiotic belonging to the polymyxin class that was introduced in the 1950s and eventually fell out of favor due to severe side effects.[10] Today, it is typically thought of as a last resort therapy for multidrug-resistant (MDR) gram-negative bacteria, and resistance to this antibiotic is rare.[10] Therefore, the discovery of the *mcr-1* plasmid, which allows horizontal transfer of colistin resistance genes, is troubling as it has the potential to open the door for truly pan-resistant microbes. With recent reports of three more cases of bacteria bearing the *mcr-1* plasmid cultured from patients in the United States,[9,11,12] resistance to colistin is a growing concern. Another recent outbreak of MDR bacteria occurred in California hospitals in association with procedures involving duodenoscopes.[13,14] There, carbapenem-resistant Enterobacteriaceae (CRE) was linked to two deaths after being transmitted by reprocessed scopes.[13,14] The carbapenem class of antibiotics is also considered to be a last resort class of antibiotics for MDR gram-negative bacteria.[15]

One cause of antibiotic resistance is the continuous evolution of bacterial genomes. Antibiotics act to kill or prevent the growth of bacteria by inhibiting crucial cellular mechanisms in the bacterium, such as protein synthesis. Inhibition of such critical functions, therefore, places tremendous stress on the organism and selects for small populations with advantageous mutations that render these compounds less active or inactive. Resistance through evolution, therefore, is a continuous process and must be countered with judicious use of the currently effective antibiotics, as well as continued development of novel therapeutics.

Another contributing factor to the rise in resistance is the overuse of antibiotics. To begin to address this issue, the White House announced a National Action Plan for Combating Antibiotic-resistant Bacteria in 2015, with a goal to reduce overuse of antibiotics by 50% in the outpatient setting, and by 20% in the inpatient setting, by 2020.[16] Globally, the World Health Organization (WHO) formed a global action plan, calling on all countries to increase awareness and knowledge surrounding antibiotic use, to improve preventative measures, to optimize the use of antibiotics in human and animal health, and to incentivize investment in novel interventions such as medicines and diagnostics.[17] While the exact number of inappropriate antibiotic prescriptions is unknown, a recent study suggested that, of the estimated 154 million annual prescriptions for oral antibiotics in the ambulatory care setting in the United States, 30% of these prescriptions were inappropriate.[18] These numbers alone are striking, and the analysis did not include hospitalized patients. Therefore, the absolute number of inappropriately prescribed antibiotics is certainly higher. Adequate antibiotic stewardship also requires determination of the correct drug and dose to use when medically indicated, as well as calls upon patients to take prescriptions as indicated. Engaging patients in these efforts to minimize the emergence of resistance will require increased education and awareness about the cause of bacterial resistance. Recent studies conducted by the Wellcome Trust and the WHO found that many people erroneously believe that patients become resistant to antibiotics, rather than the bacteria acquiring resistant genes.[1,2,19] This may lead to misuse of antibiotics, with patients believing that they should stop the course of treatment as soon as they feel better to decrease their likelihood of developing resistance to the treatment.

Finally, lack of development of novel antibiotics is another contributing factor that limits our arsenal of drugs to treat infections as they become resistant to other antibiotics. While resistance is on the rise, pharmaceutical companies have decreased their antimicrobial programs, with United States Food and Drug Administration (FDA) approval of antibiotics decreasing 56% in the 5-year period of 1998−2002 compared to 1983−1987.[20] This decline is largely due to lack of incentives, as the high costs of developing a new therapy are less likely to be recouped by an antibiotic with a short-term course of treatment.[21] In response to this, the Generating Antibiotic Incentives Now (GAIN) Act was signed into law in July 2012, giving qualified antibiotics an expedited review process and a greater number of years of exclusivity on the market.[22] Under this new act, 2014 saw the approval of four new antibiotics, only one fewer than the entire 2003−2007 period.[21] While the GAIN Act has had early success in increasing the incentives for antibiotic development, the constant process of bacterial evolution to gain resistance to antibiotics means that both novel compounds and novel classes of antibiotics need to be continuously produced to keep pace.

An alternative approach to antibiotic treatments is the development of antivirulence agents. Instead of killing the bacteria or preventing proliferation, antivirulence agents target the virulence factors that mediate disease. Antivirulence strategies provide a number of advantages over antibiotic

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Table 1. Drug Resistant Bacterial Pathogens and Their Threat Level to Society According to the Centers for Disease Control and Prevention (2013)[6]

bacterial pathogen	effects	resistance or emerging resistance
	Urgent	
Clostridium difficile	diarrhea, pseudomembranous colitis	naturally resistant to many antibiotics, fluoroquinolones
carbapenem-resistant Enterobacteriaceae spp.	UTIs, septicemia, wound infections, pneumonia	nearly all antibiotics, including carbapenems
Neisseria gonorrhoeae	gonorrhea	tetracycline, ciprofloxacin, penicillin,[284] azithromycin, cefixime, ceftriaxone
	Serious	
multidrug-resistant *Acinetobacter* spp.	pneumonia, septicemia	nearly all antibiotics, including carbapenems
drug-resistant *Campylobacter* spp.	(bloody) diarrhea, fever, temporary paralysis	ciprofloxacin, azithromycin
fluconazole-resistant *Candida* spp. (fungus)	candidemia, varies depending on site of infection	fluconazole and other azoles, echinocandins
extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae spp.	UTIs, septicemia, wound infections, pneumonia	penicillins, cephalosporins
vancomycin-resistant *Enterococcus* spp.	many, including septicemia, surgical site infections, UTIs	vancomycin
multidrug-resistant *Pseudomonas aeruginosa*	pneumonia, UTIs, septicemia, surgical site infections	nearly all antibiotics, including aminoglycosides, cephalosporins, fluoroquinolones, carbapenems
drug-resistant nontyphoidal *Salmonella* spp.	diarrhea, septicemia	multiple classes of drugs; ceftriaxone, ciprofloxacin
drug-resistant *Salmonella* serotype Typhi	diarrhea, fever, bowel perforation, shock, death	ceftriaxone, azithromycin, ciprofloxacin
drug-resistant *Shigella* spp.	(bloody) diarrhea, fever	ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, azithromycin
methicillin-resistant *Staphylococcus aureus*	many, including skin and wound infections, pneumonia, septicemia	methicillin, cephalosporins
drug-resistant *Streptococcus pneumoniae*	pneumonia, meningitis, septicemia, otitis media, sinus infections	penicillins, erythromycin and other macrolides
drug-resistant *Mycobacterium tuberculosis*	tuberculosis	isoniazid, rifampicin, fluoroquinolones, amikacin, kanamycin, capreomycin
	Concerning	
vancomycin-resistant *Staphylococcus aureus*	many, including skin and soft tissue infections, septicemia, pneumonia	vancomycin, methicillin
erythromycin-resistant Group A *Streptococcus* spp.	many, including pharyngitis, toxic shock syndrome, necrotizing fasciitis, scarlet fever, rheumatic fever, impetigo	clindamycin, macrolides, tetracycline
clindamycin-resistant Group B *Streptococcus*	many, including septicemia, pneumonia, skin infections	clindamycin, erythromycin, azithromycin, vancomycin

treatment. First, they specifically target the bacteria causing disease, in contrast to antibiotics, which kill many commensal microbes making up our healthy microbiome in addition to acting on the pathogenic bacteria. By selectively targeting the pathogen of interest, antivirulence agents do not cause collateral damage to the microbiome, and for some pathogens, a healthy microbiome may reduce the ability of the pathogen to colonize the host. Furthermore, increasing evidence suggests that a healthy microbiome is essential for the overall health of humans.[23] Dysbiosis has been linked to a diverse range of diseases, from bacterial infections such as *Clostridium difficile*, to metabolic disorders and obesity, to diseases of mental health.[23−26] Second, since virulence factors are not essential for the survival of the bacterium, bacteria may be under less selective pressure to develop resistance to these agents. Additionally, the site of action of many of these agents is outside of the bacterium itself, which may further decrease selective pressure. Third, targeting virulence also increases the number and diversity of therapeutic targets, a current challenge in the development of novel antibiotic classes. Fourth, since virulence factors, especially bacterial toxins, often mediate damage to host tissues, targeting virulence factors will reduce the morbidity of disease. Finally, these agents can be used not just as potential therapeutics, but also as chemical tools to further study the pathogenesis of disease.

It is likely that effective management of bacterial infections will require the combined use of antibiotics and antivirulence agents to reduce the bacterial burden while simultaneously reducing the pathology of disease. Antivirulence agents also have the potential to be used as prophylactic therapies for high-risk patients. For example, *C. difficile* infection usually occurs due to dysbiosis in the microbiota of the gastrointestinal system as a result of antibiotic use, allowing the pathogen to thrive. A selective antivirulence agent for *C. difficile*, therefore, could be used concurrently with antibiotics in high-risk patients.

This review discusses the rapidly developing field of antivirulence strategies to combat bacterial pathogens. We will focus on small-molecule and other chemical agents that target bacterial exotoxins, with an emphasis on both therapeutic applications and their use as tools to further elucidate mechanisms of pathogenesis. While this review focuses on AB toxins, there are other virulence factors elaborated by bacterial pathogens that can serve as potential targets for antivirulence agents, including adhesive fibers, mediators of biofilm formation, bacterial quorum sensing mechanisms, and specialized bacterial secretion systems. While these targets will not be discussed in this review, many reviews discuss the progress made on these topics.[27−30] Finally, we will discuss challenges in the development and advancement of targeted antivirulence therapies to human clinical trials.

2. HOW DO BACTERIAL AB TOXINS WORK?

This review focuses on the inhibition of exotoxins, or toxins excreted from the bacterium, that exert their function in the

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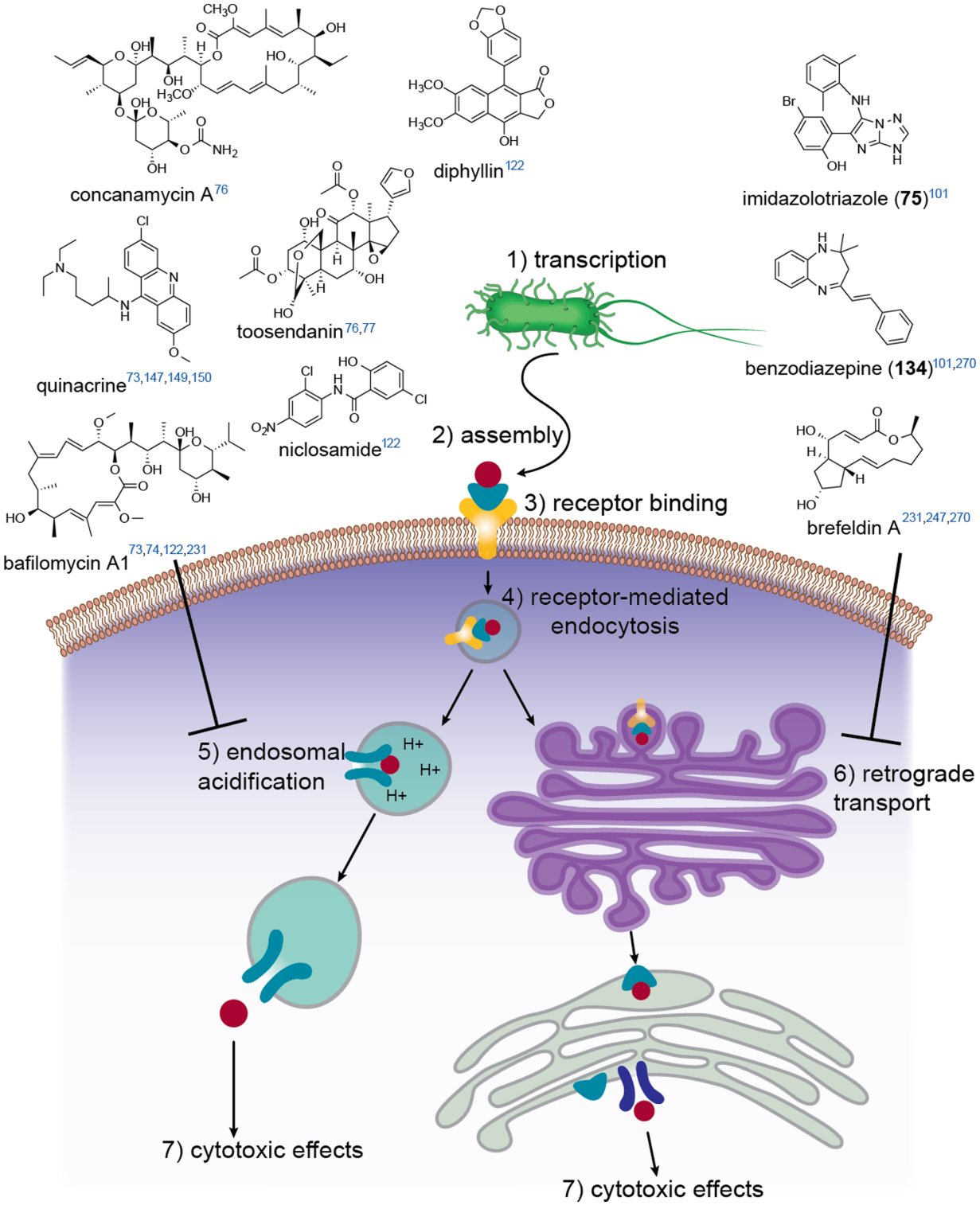

Figure 1. Schematic of toxin production, internalization into host cells, and steps targeted by small-molecule antivirulence agents. Toxins comprised of an active "A" subunit (red) and binding "B" subunit (blue) are transcribed in the bacterium (1), assembled (2), and bind to cell-surface receptors (yellow) (3). Upon receptor-mediated endocytosis (4), toxins follow one of two pathways: classical endocytosis or retrograde transport. In classical endocytosis, endosomes containing toxin are acidified (5), mediating extrusion of the active subunit into the cytosol to exert its cytotoxic effect on host protein targets (7). In the retrograde pathway, toxins are trafficked from endosomes to the trans-Golgi network and then to the endoplasmic reticulum (6), where host pores (purple) mediate extrusion of the active subunit into the cytosol to exert its cytotoxic effect on host protein targets (7). The small-molecule inhibitors acting at steps 5 and 6 included in this figure inhibit multiple toxins discussed in this text.

cytosol of host cells. Additionally, many of the toxins discussed here are structurally classified as AB toxins, which have an active "A" subunit and a binding "B" subunit. From transcription of the toxin gene to blocking the cytotoxic effects of the toxin in the host cell, there are many steps that can be targeted by small-molecule antivirulence agents. Additionally,

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because some internalization steps are conserved for multiple toxins, small-molecule inhibition at these steps can sometimes block more than one toxin. An overview of the various stages a toxin must go through to reach the host cytosol and induce cellular damage is shown in Figure 1.

The first step that can be inhibited by small-molecule inhibitors is transcription of the virulence factor (Figure 1, step 1), a strategy that has mostly been utilized by inhibitors targeting cholera toxin (see section 3.3.1). Inhibiting toxin production is attractive because the inhibitor works on the bacterium itself rather than in the host cells, making it possible to minimize cross reactivity or host toxicity. However, because these agents act within the bacterium itself, there may be greater selective pressure to develop resistance mutations or efflux pumps to reduce the efficacy of these compounds. Additionally, delivery can be a challenge depending on the site of colonization of the bacterium. Once translated, toxins must be correctly assembled (Figure 1, step 2). This process varies for each specific toxin, from no assembly for some toxins synthesized as a single polypeptide, to the case of anthrax toxin, which requires proteolytic cleavage by extracellular host proteases before assembly into a multimeric complex on the cell surface. Due to the complex nature of anthrax toxin assembly, it is perhaps not surprising that most of the small-molecule inhibitors of toxin assembly have been developed against anthrax (see section 3.8.1). Another step that is unique for each specific toxin is receptor binding on the host cell (Figure 1, step 3). For toxins for which the host cell receptor is known, receptor mimics are a popular strategy to inhibit toxin entry into the cell, and this review highlights numerous examples of such compounds. In general, inhibiting toxin assembly and binding are attractive options because compounds do not need to be permeant to the host cell, a significant challenge for many small-molecule inhibitors. However, because these compounds target early stages of toxin production, their efficacy may decrease later in the infection course after large amounts of toxin have already been synthesized and internalized. This may be a significant drawback of these inhibitors, especially with rare infections that are difficult to diagnose early in the course of infection.

After receptor binding, all toxins are internalized by receptor-mediated endocytosis (Figure 1, step 4). Here, toxins take one of two routes: anthrax, *C. difficile*, and botulinum toxins follow the typical endocytic pathway (Figure 1, step 5), while Shiga and cholera toxins are trafficked through retrograde transport (Figure 1, step 6). In the first route, acidification of endosomes induces conformational changes that mediate extrusion of the A subunits of the toxin into the host cytosol. Because many of the critical proteins involved in endosome acidification are host-derived, small-molecule inhibitors at this step often affect multiple toxins. The small-molecule inhibitors illustrated in Figure 1, step 5 inhibit multiple toxins and will be referenced later in the text. In retrograde transport, endosomes containing toxin are trafficked to the trans-Golgi network and then the endoplasmic reticulum (ER), where the toxins utilize host channel proteins to enter the cytosol. Retrograde transport inhibitors can also block multiple toxins, likely due to their host targets (Figure 1, step 6). For both endosome acidification and retrograde transport inhibitor approaches, a downside is that these compounds typically target host proteins or membranes instead of directly binding the toxin, leading to the possibility of host toxicity. However, they also have multiple benefits, including the ability to target multiple toxins, and their potential use for bacterial infections of unknown etiology.

Finally, the most common class of small-molecule antivirulence inhibitors target the active "A" domains of toxins (Figure 1, step 7). These compounds are typically specific for one toxin, although there are examples of antivirulence agents that inhibit multiple toxins, usually when the toxins are of the same functional enzyme class and the inhibitors are substrate-based. Compounds targeting toxin function are desirable because they directly block the active portion of the toxin that causes cellular damage. Additionally, these compounds can be more specific for the toxin of interest, decreasing cross reactivity with the host and other bacteria. Finally, these inhibitors are likely to be efficacious when given at any point during infection, as they act at the final step of toxin action. Drawbacks to this approach include the requirement for host cell permeability and challenges in delivery to target tissues. In addition, since these compounds are specific for one toxin and meant to be taken for a short and curative treatment course, incentives for their development may be lower.

3. SMALL-MOLECULE ANTIVIRULENCE AGENTS

3.1. *Clostridium difficile*

C. difficile is a gram-positive, anaerobic, opportunistic pathogen that is the leading cause of hospital-acquired infections.[31] Interestingly, the CDC lists *C. difficile* in the urgent threat category (Table 1) not because of resistance to the antibiotics used to treat *C. difficile* infection (CDI), but due to its natural resistance to many other antibiotics used to treat unrelated conditions. In fact, antibiotics are the leading and most modifiable risk factor for the development of CDI, and therefore, CDI is a direct consequence of the overuse and misuse of antibiotics. Infection with *C. difficile* usually occurs when patients are placed on antibiotics for an unrelated infection. As collateral damage, the microbiome in the gastrointestinal tract is altered, allowing this opportunistic pathogen to colonize. Symptoms of CDI can range from diarrhea and abdominal discomfort to pseudomembranous colitis, toxic megacolon, and even death. Incidence of CDI is rising; in the United States alone, approximately 250 000 patients required hospitalization for CDI, and it directly causes 14 000 deaths per year.[6,32] To try to stem the rise in CDI, the White House issued a goal to decrease incidence of CDI by 50% from 2011 levels by the year 2020.[33]

CDI is mediated by two main virulence factors: the large clostridial toxins TcdA (308 kDa) and TcdB (270 kDa). TcdB is considered the primary virulence factor in human infection,[34−36] with most strains recovered from infected patients expressing TcdB.[37] While atoxigenic strains can also colonize humans, they do not cause clinical disease.[38] This observation has led to one nonantibiotic strategy for treatment of CDI in which patients are given atoxigenic strains of *C. difficile* to occupy the optimal niche, thus preventing colonization by pathogenic strains. In phase II clinical trials, patients were treated with either nontoxigenic *C. difficile* strain M3 (NTCD-M3, also called VP20621) or placebo after achieving clinical cure with antibiotic treatment (clinicaltrials.gov identifier NCT01259726).[39] The study achieved the primary end point of safety and tolerability, and also significantly decreased recurrence rates for all doses and treatment regimens of NTCD-M3 as compared to placebo (11% for NTCD-M3 compared to 30% for placebo, p =

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0.006).[39] However, one serious concern for this approach lies in the fact that the genes encoding TcdA and TcdB are located on a chromosomal region called the pathogenicity locus (PaLoc) that can be transferred from toxigenic to atoxigenic strains.[40] After acquiring the PaLoc, newly toxigenic strains are capable of producing toxin.[40] This finding leads to the concerning possibility that virulence could be transferred from pathogenic strains to the atoxigenic treatment strain.

TcdA and TcdB are multidomain exotoxins each consisting of an N-terminal cytotoxic glucosyltransferase domain (GTD), a cysteine protease domain (CPD), a transmembrane domain, and a C-terminal receptor binding domain.[41] TcdA and TcdB share 66% sequence identity, which increases to 74% on the N-terminus bearing the GTD. This high homology is thought to come from a gene duplication event.[42] Excreted toxins are taken up into enterocytes by receptor-mediated endocytosis.[43] Upon acidification in late endosomes, the transmembrane domain mediates extrusion of the CPD and GTD into the cytosol of the host cell, where the CPD is allosterically activated by the mammalian-specific sugar 1D-*myo*-inositol hexakisphosphate (IP_6).[41] The activated CPD autoproteolytically cleaves after leucine at amino acid residue 542 (TcdA) or 543 (TcdB), releasing the GTD to glucosylate members of the Rho/Rac family of GTPases. Monoglucosylation of these GTPases in the host cell disrupts the actin cytoskeleton and leads to rapid cell death.[44,42]

While antibiotics are the number one most modifiable risk factor associated with CDI, paradoxically, the current gold standard treatment for CDI is also antibiotics. Vancomycin, metronidazole, and the recently approved fidaxomicin are commonly prescribed antibiotics to treat CDI. Initial cure rates with metronidazole or vancomycin are 72.7−81.1%,[45] and 15−25% of patients will have a recurrence of CDI.[46] After the first recurrence, approximately half of patients will have subsequent recurrences.[46] Antibiotic treatment that precludes recovery of the healthy microbiome is a major contributing factor to high recurrence rates. Despite this, none of the currently FDA-approved therapies for CDI spare the healthy microbiome or allow its recovery. In contrast, antivirulence agents that specifically target TcdA and TcdB would mitigate the morbidity of CDI while allowing the healthy microbiome to recover, thereby restoring gut homeostasis and allowing commensal flora to naturally control *C. difficile* colonization. This approach has been validated by Merck, which gained FDA approval in October 2016 for their monoclonal antibody, bezlotoxumab (Zinplava), which neutralizes TcdB.[36] The antibody binds to specific combined repetitive oligopeptide (CROP) regions found within the C-terminal receptor binding domain of the toxin,[47] preventing toxin binding to cell surface receptors. When treating patients with bezlotoxumab in addition to standard of care antibiotics, recurrence rates were reduced from 25 to 7% compared to placebo in a phase II clinical trial and from 27.6 to 17.4% in the phase III MODIFY I clinical trial (clinicaltrials.gov identifiers NCT00350298, NCT01241552, and NCT01513239).[36,48] Interestingly, no benefit was seen when treating with the monoclonal antibody targeting TcdA, actoxumab, over placebo-treated controls, nor did the combination bezlotoxumab/actoxumab treatment benefit patients any more than bezlotoxumab alone.[36] This suggests that TcdB is the primary virulence factor causing pathology of CDI in humans. Further, this clinical trial confirmed the benefits of using antivirulence agents in combination therapy with standard of care treatments. However, the high cost of production and intravenous (IV) route of administration for monoclonal antibodies limit the utility of antibody-based treatments for use in patients with community acquired CDI or as prophylaxis for high-risk patients beginning antibiotic treatments. Therefore, in addition to these monoclonal antibodies, several small-molecule approaches have been pursued to develop novel nonantibiotic therapies for CDI.

3.1.1. *C. difficile*: Ion-Exchange Resins. Ion-exchange resins that bind to and neutralize TcdA and TcdB in the gastrointestinal system were the first nonantibiotic strategies tested for the treatment of CDI. After anecdotal clinical reports were published describing the efficacy of cholestyramine in patients with antibiotic-associated pseudomembranous colitis,[49] Chang and colleagues showed that two anion-exchange resins, cholestyramine and colestipol, protected against cytotoxicity in a cell-based assay in both a time- and concentration-dependent manner.[50] While colestipol was able to reduce toxin titers to undetectable levels, cholestyramine reached saturation before neutralizing all active toxin, leading the authors to conclude that colestipol was 4 times more potent per weight than cholestyramine. Further studies indicated that both resins bound to toxin in vitro.[51] Furthermore, in a hamster model of CDI in which infection with *C. difficile* is uniformly fatal, treatment with cholestyramine delayed mortality; however, treatment was inferior to protection from vancomycin.[51] Another drawback of this treatment revealed by in vitro experiments was that the resins also bind to vancomycin, a significant hurdle for cotreatment options in patients. This was also shown in the hamster model of CDI, where hamsters in a cholestyramine + vancomycin group died sooner than the vancomycin-treated groups, perhaps due to binding of vancomycin to the anion-exchange resin. While human clinical trials were never initiated for the treatment of CDI, both cholestyramine and colestipol are FDA-approved drugs, and historical evidence exists for their off-label use to treat CDI.[49,52]

Another ion-exchange resin, tolevamer (also known as GT160-246 and GT267-004), was tested in animal models and human clinical trials for efficacy in the treatment of CDI (clinicaltrials.gov identifiers NCT00382304, NCT00034294, NCT00466635, NCT00106509, and NCT00196794). In the hamster model of CDI, 70−90% of tolevamer-treated hamsters survived at doses between 500 and 1500 mg/kg per day, compared to a 10% survival for saline-treated hamsters.[53] In a comparison of 1000 mg/kg per day tolevamer and 1000 mg/kg per day cholestyramine using a hamster model of CDI where treatment was prophylactically started 2 days before infection, 80% of hamsters in the tolevamer treatment arm survived, compared to a 10% survival of cholestyramine-treated hamsters.[53] This study showed the improvement of tolevamer over the previous generation of ion-exchange resins. Tolevamer was then tested in a phase II noninferiority clinical trial as a monotherapy compared to vancomycin. Patients received 3 or 6 g of tolevamer per day or the standard of care treatment of 500 mg of vancomycin per day, and noninferiority was achieved for the primary end point of time to resolution of diarrhea for the 6 g tolevamer arm (2.5 days) as compared to vancomycin treatment (2.0 days) ($p = 0.02$).[54] As a secondary outcome, the 6 g tolevamer group had a 7% recurrence rate compared to 19% for vancomycin treatment, a trend that narrowly missed achieving statistical significance with $p = 0.05$.[54] Despite the success of the phase II trial, tolevamer was found to be inferior to vancomycin in two identical phase III clinical trials (clinicaltrials.gov identifiers NCT00106509 and

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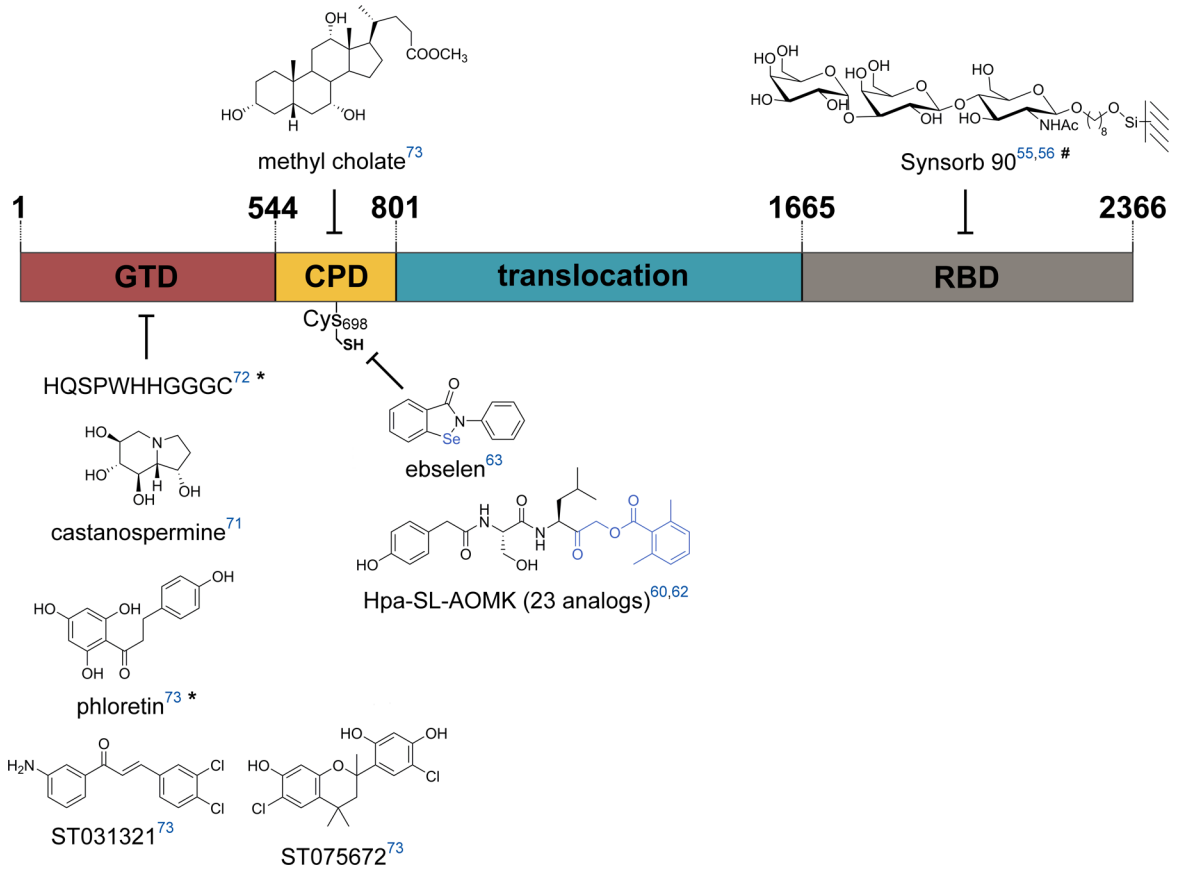

Figure 2. Inhibitors targeting various domains of TcdB, one of the two major virulence factors of *C. difficile*. Phloretin and castanospermine target the glucosyltransferase domain (GTD; red) by mimicking the structure of the substrate sugar. The peptide sequence HQSPWHHGGC also binds to the GTD. Ebselen and Hpa-SL-AOMK target the cysteine protease domain (CPD; yellow) by covalent modification of the active site cysteine through the electrophilic selenium or acyloxymethyl ketone groups (blue), while methyl cholate targets the CPD by an unknown binding mode. Synsorb 90 was hypothesized to neutralize TcdA but not TcdB (#) by binding to the receptor binding domain. Compounds found to be active against TcdA and TcdB are indicated (∗).

NCT00196794) measuring clinical success as the primary end point.[45] Patients were treated with a 9 g loading dose followed by 3 g every 8 h for 14 days, or one of two standard of care antibiotics: vancomycin or metronidazole. In the tolevamer-treated groups, 44.2% of patients achieved clinical success compared to 72.7% for metronidazole treatment and 81.1% for vancomycin-treated patients ($p < 0.001$ for comparisons between tolevamer and metronidazole, and between tolevamer and vancomycin).[45] These unsuccessful phase III trial results halted further efforts to bring tolevamer to market.

Another toxin binder, Synsorb 90 (or Synsorb-CD), was developed by Synsorb Biotech as an inert silica-based support containing the trisaccharide receptor binding motif for toxin A found on enterocytes, Gal(α1−3)Gal(β1−4)GlcNAc (Figure 2).[55,56] Synsorb was hypothesized to bind to and neutralize toxin A but not affect toxin B.[55] In a rat ileal model with purified toxin A, pretreatment with Synsorb 90 protected against intestinal secretion, mucosal damage, edema, inflammation, and neutrophilic infiltration.[57] In a phase II human clinical trial, patients with one recurrence of CDI were initially treated with metronidazole before randomization to receive placebo, 8 g per day Synsorb 90, or 16 g per day Synsorb 90 for 24 days and monitored for recurrence of CDI.[58] The phase II trial was halted at interim analysis due to overwhelmingly positive results at the high dose, in which recurrence rates were 41.7, 36.7, and 21.9% for placebo treated, low dose Synsorb 90 treated, and high dose Synsorb 90 treated groups, respectively.[58] Despite these positive phase II interim results and receiving Fast Track designation by the FDA for the treatment of recurrent CDI, Synsorb Biotech abandoned development of the decoy receptor due to low enrollment and high dropout rates in phase III clinical trials.[58,59]

3.1.2. *C. difficile*: Inhibitors of the Cysteine Protease Domain. In contrast to the first generation antitoxin agents, newer strategies have focused on directly targeting and inactivating toxin domains. The first study to show that the CPD was a druggable target for the treatment of CDI used a focused library of rationally designed peptidic small molecules based on the substrate recognition site of the cysteine protease of TcdB.[60] Using the natural cleavage site of this clan CD protease, leucine 543, in the P1 position, structure−activity relationship (SAR) analyses of dipeptide acyloxymethyl ketone (AOMK) inhibitors revealed that smaller P2 residues and a hydroxy-phenyl acetyl (Hpa) cap increased potency. The AOMK was chosen as a warhead because it is highly specific for cysteine proteases while remaining inactive toward other general nucleophiles.[61] Inhibition occurs through nucleophilic attack of the cysteine sulfur on the ketone-carbonyl followed by migration and loss of the *O*-acyl group to generate a stable thiol ester linkage. The most potent compound, Hpa-SL-AOMK, contained the two natural amino acid residues of the CPD substrate and had an IC_{50} of 0.71 μM in a gel-based

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autocleavage assay (Figure 2).[60] This inhibitor scaffold was further developed into two activity-based probes (ABPs), with AWP-19 containing a Cy5 fluorophore, and AWP-15 containing a biotin affinity purification tag. These probes were used to monitor the activity of the CPD, as they do not bind to the nucleophilic cysteine residue until the CPD is activated by IP_6. This ABP then was used to uncover the mechanism of allosteric activation of the CPD by IP_6. In-gel fluorescence labeling with AWP-19 revealed that the apo-CPD was activated in the absence of IP_6 in a time-dependent manner, indicating that the protease could convert to the active confirmation without allosteric activation.[62] Further, AWP-19 was used to determine the EC_{50} concentration of IP_6 on protease activity as 0.17 μM. These studies highlight the utility of small-molecule inhibitors not only as potential therapeutics, but also as tools to further characterize the mechanism of toxin action.

The probe AWP-19 containing a carboxytetramethylrhodamine (TAMRA) fluorophore (TAMRA-AWP-19) was further used in a fluorescence polarization high-throughput screen (FP HTS) of over 130 000 compounds to identify small-molecule drug leads targeting the CPD.[63] This screen identified ebselen (2-phenyl-1,2-benzoselenazol-3-one), a compound currently in phase II clinical trials for hearing loss and Meniere's disease (Sound Pharmaceuticals) (clinicaltrials.gov identifiers NCT01452607, NCT02819856, NCT01444846, NCT01451853, NCT02779192, and NCT02603081), as a potent inhibitor of the CPD (Figure 2).[64] A known clinical compound with a clean human safety profile, ebselen is reported to have antioxidant-like properties and function in a glutathione peroxidase-like manner through its selenium atom.[65,66] Ebselen was found to covalently bind to the active site cysteine residue, inhibit CPD autoprocessing of both TcdA and TcdB in vitro, and protect against TcdB-mediated cytotoxicity in cell-based assays with low nanomolar potency. Importantly, in a mouse model of CDI, treatment with ebselen protected against toxin-mediated pathology in colon tissues in a dose-dependent manner, with 100 mg/kg per day treatment achieving histological scores indistinguishable from uninfected controls. Further, Western blot analysis of colonic tissues confirmed a dose-dependent inhibition of CPD-mediated GTD processing, implying target engagement of ebselen with the CPD in host tissues. Interestingly, cell-based studies with TcdA and TcdB mutants have indicated that disruption of autocleavage decreases potency and delays toxicity but does not completely abolish cytotoxicity.[67−70] However, whether this delayed and attenuated toxicity is relevant in the context of in vivo infection is unclear. Thus, to fully assess whether CPD inhibition is sufficient for protection against toxin-mediated pathology in vivo, studies with *C. difficile* mutants will need to be performed in clinically relevant animal models of disease. These studies have not yet been carried out due to the difficulty of genetically manipulating this bacterium.

3.1.3. *C. difficile*: Inhibitors of the Glucosyltransferase Domain. In addition to targeting the CPD, several studies have identified inhibitors of the glucosyltransferase domain (GTD). In a study primarily focused on finding inhibitors for the related clostridial species *Clostridium sordellii*, Jank and colleagues identified the plant alkaloid castanospermine as a GTD inhibitor with an in vitro IC_{50} of 400 μM (Figure 2).[71] Structural analysis revealed that castanospermine functions by acting as a transition state mimic. While the low potency of castanospermine likely precludes its development as a drug lead, this study showed that substrate mimics of glucosyltransferases could be used to inhibit multiple clostridial glucosylating toxins. Another study used phage display to identify GTD inhibitors.[72] Two peptides with the most potent binding affinities, EGWHATGGGC and HQSPWHHGGGC, were synthesized and tested in vitro (Figure 2). Using radiolabeled UDP-glucose, EGWHAHTGGGC inhibited TcdA glucosyltransferase activity with a K_i of 500 nM while HQSPWHHGGGC was slightly more potent with a K_i of 300 nM. Both peptides were more potent against TcdB, inhibiting glucosyltransferase activity with K_i values of 54 nM for EGWHAHTGGGC and 18 nM for HQSPWHHGGGC. Docking studies showed the peptides coordinating with the catalytic magnesium ion, highlighting one potential strategy for targeting the GTD domain.

3.1.4. *C. difficile*: General Antitoxin Strategies. In another example of efforts to find small-molecule modulators of toxin function, Tam and colleagues used a high content cell-based screen to identify small molecules that blocked TcdB-induced cellular damage.[73] In concurrence with a previous report,[74] small molecules that inhibited acidification of endosomes neutralized TcdB cytotoxicity. Protection against TcdB-induced cell rounding by bafilomycin A1, a v-ATPase inhibitor, and quinacrine, an antimalarial drug, closely correlated with their ability to block the acidification of endosomes necessary for toxin translocation into the cytosol of host cells (Figure 1).[73,74]

In the same screen, the bile acid analogue methyl cholate was found to bind to TcdB, but not TcdA, and neutralize the toxin via conformational stabilization (Figure 2).[73] TcdB had an increase in thermal stability, a dose-dependent reduction of CPD-induced autoprocessing, and a decrease in its ability to bind the host cell receptor in the presence of methyl cholate. Interestingly, the decrease in receptor binding obtained with 40 μM methyl cholate was similar to that of 1.4 μM bezlotoxumab. Given the result that only the TcdB-neutralizing antibody bezlotoxumab is necessary to protect against recurrence in human clinical trials, the similar efficacy between methyl cholate and bezlotoxumab suggests that methyl cholate should be sufficient to block toxin-mediated pathology in human CDI.

The flavanoid phloretin was another hit from the high content cell-rounding screen, and was found to be a noncompetitive inhibitor of the GTD (Figure 2).[73] Phloretin inhibited both glucosyltransferase activity of the GTD to its substrate Rac1 and hydrolysis of UDP-glucose using a commercial glycosyltransferase assay, with IC_{50} values of 2.1 and 207.1 μM, respectively. With this flavanoid hit, Tam and colleagues further screened a restricted library of 500 flavanoid analogues and found two other promising hits. ST031321 partially protected against TcdA- and TcdB-mediated cell rounding, and ST075672 offered complete protection (Figure 2). ST075672 also inhibited glucosyltransferase activity and UDP-glucose hydrolysis, with IC_{50} values of 7.7 and 38.9 μM, respectively, showing improvement in potency over the original hit.

The small molecules identified in this study[73] were then further used to study the mechanism of NADPH-oxidase mediated necrosis, a phenomenon of toxicity induced at 100−1000-fold higher concentrations of TcdB than the concentration required for cell rounding.[67,75] Quinacrine and methyl cholate protected cells against TcdB-mediated necrosis with IC_{50} values of 1.6 and 4.3 μM, respectively, while phloretin did not protect cells (Figures 1 and 2).[73] This result helped confirm

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the finding that pore formation, inhibited by the endosomal acidification inhibitor quinacrine, and receptor binding, inhibited by the conformational stabilizer methyl cholate, are important aspects of TcdB-induced necrosis.[73] However, necrosis is thought to be CPD- and GTD-independent, consistent with the result that the GTD inhibitor phloretin did not protect cells.

In a study primarily aimed at finding inhibitors for anthrax, Slater and colleagues performed a phenotypic screen to identify small molecules capable of protecting cells from lethal toxin (LT)-induced macrophage cell death.[76] Many of the compounds identified also inhibited TcdB-induced cell death. The terpene toosendanin, a natural product from the plant *Melia azedarach* var. *toosendan* already known to inhibit botulinum toxin in vivo,[77] was found to inhibit TcdB-induced cytotoxicity (Figure 1). This compound, as well as another inhibitor of endosomal acidification concanamycin A, is hypothesized to act on multiple toxins by interfering with cellular internalization (Figure 1). Targeting the toxin internalization pathway is one strategy that may lead to antivirulence agents capable of acting on multiple bacterial pathogens. Interestingly, many FDA-approved drugs such as pizotifen malate, tilorone, and disulfiram were also identified as inhibitors of TcdB-induced cytotoxicity. However, the potencies of these compounds were not reported and the mechanisms of inhibition are unknown, requiring significant follow-up before the compounds reported in this study could be considered therapeutic leads.

Small-molecule inhibitors of TcdA and TcdB have significant potential to advance to human clinical trials and FDA approval. First, the strategy to target *C. difficile* toxins was proven to be a successful approach by the recent FDA approval of bezlotoxumab in October 2016. Second, many of the compounds discussed are highly potent for their targets, giving them promise to move forward to preclinical animal models and human clinical trials. Further, due to the large numbers of high-risk patients—those taking antibiotics or with previous CDI—there is a need for small-molecule inhibitors targeting TcdA and TcdB to be used as prophylactic agents.

3.2. *Clostridium sordellii*

Another clostridial species that causes disease in humans is *C. sordellii*. The two major virulence factors of *C. sordellii* are the exotoxins lethal toxin (TcsL) and hemorrhagic toxin (TcsH). TcsL acts in the same manner as TcdA and TcdB of *C. difficile*, irreversibly glucosylating small Rho/Rac GTPases, leading to actin cytoskeletal dysfunction and cell death. However, glucosylation activity of TcsL is more promiscuous than TcdA and TcdB, with TcsL also capable of glucosylating Ras family proteins. Infection is rare, with virulent strains of *C. sordellii* capable of causing gangrene, myonecrosis, pneumonia, endocarditis, arthritis, and peritonitis.

3.2.1. *C. sordellii*: Inhibitors of the Glucosyltransferase Domain. To mitigate pathology associated with this infection, Jank et al. identified inhibitors of the effector glucosyltransferase domain (GTD).[71] A screen of azasugars and other derivatives of known glycosidase inhibitors tested against TcsL of *C. sordellii* and toxin B of *C. difficile* identified the plant alkaloid castanospermine (see Figure 2) as an inhibitor with an in vitro IC_{50} of 400 μM. Cell-based assays also showed protective effects of castanospermine against TcsL and TcdB challenge, but only at high doses of 1 mM and when injected into cells, indicating potential limitations in cell permeability. Structural studies of TcsL and castanospermine revealed that the inhibitor acts as transition state mimic to inhibit the glucosylation activity of the toxin. While optimization of the castanospermine scaffold may lead to more potent inhibitors that could be used to treat multiple clostridial glucosylating toxins, the modest potency and lack of cell permeability of this compound likely precludes it from future development.

In another study, Schulz and colleagues identified an approved drug, tauroursodeoxycholic acid (TUDCA), as an inhibitor of TcsL-induced cytotoxicity.[78] A known antiapoptotic drug that activates phosphatidylinositide 3′-OH kinase (PI3K)/Akt signaling, high doses of TUDCA (300 μM) given up to 1 h post-toxin challenge protected against TcsL-induced cytotoxicity, but did not protect against TcdA-induced cytotoxicity, suggesting that the apoptotic effect of TcsL is mediated through its ability to glucosylate Ras family proteins. Further in vitro analysis showed that TUDCA rescued TcsL-induced Ras inhibition by increasing phosphorylation of the downstream effector Akt in a PI3K-dependent manner. While TUDCA lacks potency and does not directly target TcsL, its use here as a pharmacologic tool revealed a difference in the induction of apoptosis by TcsL of *C. sordellii* compared to TcdA and TcdB of *C. difficile*.

Very few compounds have been tested as inhibitors of *C. sordellii* virulence factors. Due to the high degree of structural and functional similarity between the glucosylating domains of TscL and *C. difficile*'s TcdA and TcdB, inhibitors of the GTD of TcdA and TcdB are likely to be promising lead scaffolds.

3.3. *Vibrio cholerae*

V. cholerae is a gram-negative bacterium responsible for an estimated 1.4−4.3 million yearly infections worldwide.[79,80] A disease all but eradicated in industrial nations, cholera is still a significant worldwide killer, causing an estimated 28 000−142 000 deaths per year in areas without clean water and sanitation infrastructure.[79,80] Although classically associated with contaminated water, cholera can also be spread through undercooked food.[81] This acute diarrheal infection often affects marginalized populations, exacerbating conditions in refugee camps and after natural disasters.[82] Cholera infection causes watery diarrhea and vomiting that can lead to death from profound dehydration and shock, but is treatable in up to 80% of cases with oral rehydration salts.[80] Only severely dehydrated patients require intravenous rehydration, and antibiotics are reserved for only the sickest patients to decrease the bacterial load while minimizing emerging resistant strains.

Due to the largely supportive care provided for cholera infections and the reluctance to use antibiotics for all but the most severe cases, alternate therapies that mitigate toxin action and thereby reduce symptom severity would act as a complementary treatment that could greatly improve the supportive care provided to patients. Further, since infections largely occur in areas of economic, political, and natural disasters, effective treatments would need to be stable at room temperature for stockpile, shipping, and delivery to remote and infrastructure-poor areas. For these reasons, small-molecule inhibitors of toxin action are ideal therapies for further development.

Over 200 serotypes of *V. cholerae* have been identified, but only two cause epidemic disease: O1 and O139.[82] Serogroup O1 has been responsible for all seven pandemics, but the newly emerging O139 is predicted to be the cause of the next (eighth) pandemic.[81,82] The symptoms of cholera are mediated by the virulence factor cholera toxin (CT, also referred to as

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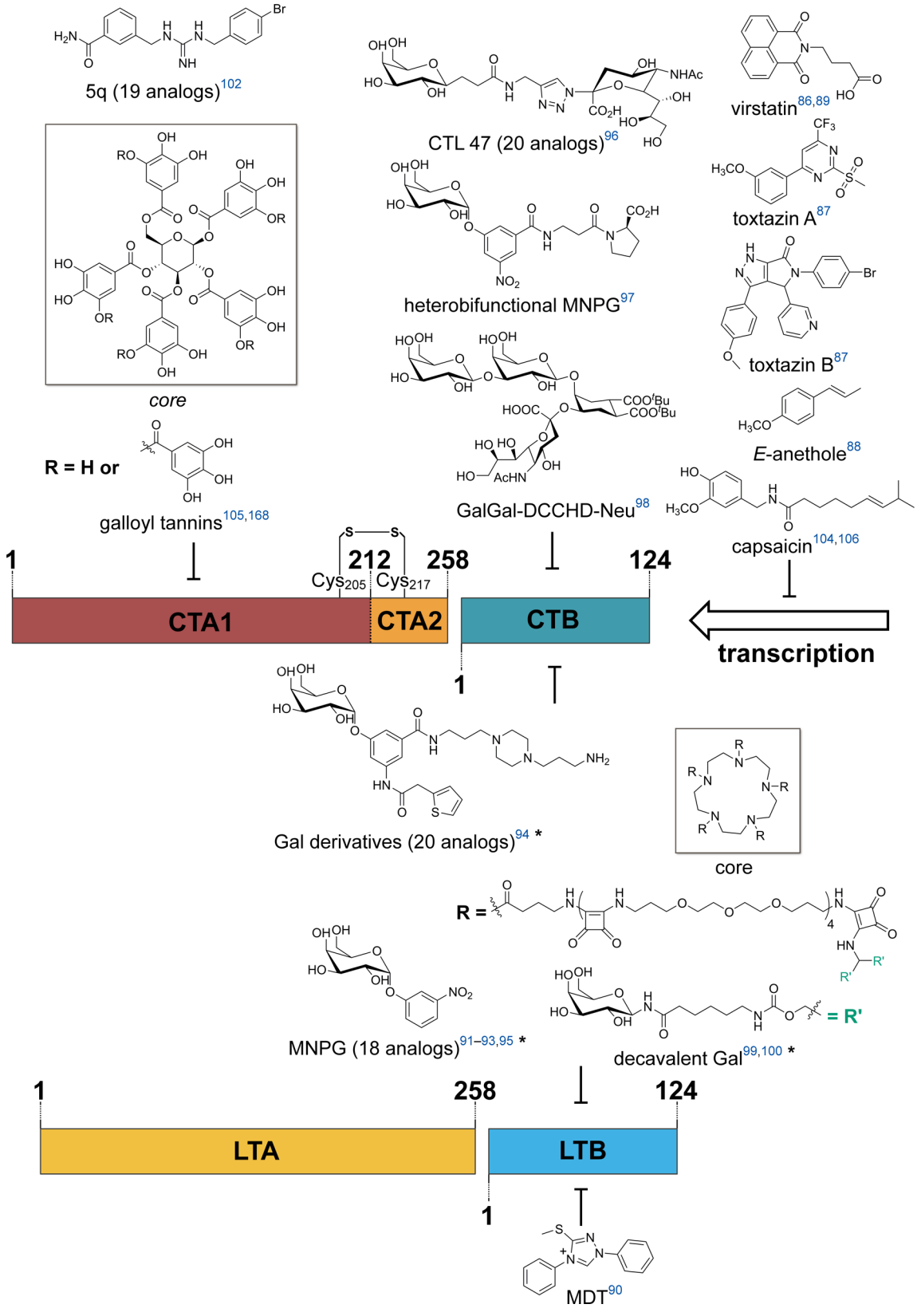

Figure 3. Compounds targeting cholera toxin (CT) of *V. cholerae* and heat-labile enterotoxin (LT) of enterotoxigenic *E. coli* (ETEC). Upper panel: Small-molecule inhibitors of CT. Upper left: Benzylguanidines (such as 5q) and galloyl tannins target the cytotoxic CTA1 subunit (red). Upper right: Small-molecule inhibitors of CT transcription. Middle column: Sugar-based compounds such as *m*-nitrophenyl-α-D-galactopyranoside (MNPG) target the B subunit (blue) of CT and LT. Due to the success of sugar-based inhibitors, other host cell receptor mimics and multivalent compounds that mimic the toxin B_5 structure have been reported. Compounds found to be active against CTB and LTB are indicated (∗). Lower panel: Small-molecule inhibitors of LT.

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choleragen and CTX), a member of the AB_5 toxin family.[82] CT is composed of an A subunit and five B subunits and exerts its toxicity once inside the small intestinal epithelium. CT first binds to the GM1 ganglioside and is taken up via endocytosis before undergoing retrograde transport through the Golgi apparatus and endoplasmic reticulum (ER).[82] Before entry into the ER, the A subunit is cleaved into A1 and A2 fragments, and the A1 fragment (CTA1) is transported to the cytosol by an unknown mechanism, where it exerts its cytotoxic effects.[82] CTA1 is allosterically activated by a host ADP-ribosylation factor ARF6, allowing it to bind NAD^+ in its active site and constitutively activate G_s α-subunit ($G_{\alpha s}$) proteins via ADP-ribosylation.[83] Activated $G_{\alpha s}$ increases adenylyl cyclase activity, increasing levels of the second messenger cyclic adenosine monophosphate (cAMP) by activating membrane-bound forms of the human cyclase up to 50-fold.[84,85] The cystic fibrosis transmembrane conductance regulator (CFTR) channel is activated by cAMP to allow chloride ions into the gut lumen, causing subsequent efflux of water, Na^+, K^+, and HCO_3^- into the lumen. Such rapid loss of water and electrolytes from the intestinal epithelium creates the characteristic rice−water stool of cholera infection.

3.3.1. *V. cholerae*: Inhibitors of Toxin Transcription. Many of the antivirulence agents developed for *V. cholerae* inhibit transcription of CT. Transcription of CT and a second virulence factor, toxin coregulated pilus (TCP), which is important for attachment and colonization, are regulated by the master virulence regulator, ToxT.[86−88] Hung and colleagues performed a high-throughput screen (HTS) of 50 000 small molecules to identify inhibitors of virulence factor expression using an engineered bacterium with an antibiotic selection cassette integrated into the genome under the *ct* promoter.[86] Among the inhibitors identified, 15 did not confer toxicity to the bacteria. One compound chosen for further study, 4-[*N*-(1,8-naphthalimide)]-*n*-butyric acid (virstatin), was shown to post-transcriptionally inhibit ToxT, completely blocking CT production in vitro at 50 μM without inhibiting bacterial growth (Figure 3). In an infant mouse model of cholera infection, virstatin inhibited colonization of *V. cholerae* in a TCP-dependent manner and eliminated the competitive advantage of wild-type bacteria over mutant strains lacking TCP. Finally, virstatin was able to reduce bacterial load even when mice were treated 12 h postinfection, demonstrating its potential use in a clinically relevant scenario in which patients already infected with cholera seek treatment. Further work showed that virstatin prevented dimerization of ToxT, thereby preventing CT production.[89] Using virstatin as a tool to study ToxT transcriptional activity, the authors found that some promoters of ToxT activation, such as *ctxAB*, *tcpA*, *acfD*, and *tagA* were sensitive to virstatin-induced prevention of ToxT dimerization. However, a second set of promoters, *tcpI*, *acfA*, and *aldA*, were less repressed by virstatin, suggesting that some promoters may not require ToxT dimerization for activation.

In a second HTS to identify small-molecule inhibitors of *toxT* transcription, 63 300 compounds were screened using an engineered *V. cholerae* with the *toxT* promoter driving the expression of GFP.[87] Among hits that reduced GFP production without killing bacteria, three compounds, toxtazins A, B, and B′, were identified as the most potent compounds in the screen with the lowest levels of CT production (Figure 3). While toxtazin A's exact mechanism of action remains unclear, using a targeted approach to identify where in the regulatory cascade each of these compounds acted, the authors found that toxtazins B and B′ significantly decreased protein levels of TcpP, one of the two major transcriptional activators of *toxT*, by inhibiting transcription of *tcpP* at its promoter. Using an infant mouse model of colonization, toxtazin B, but not toxtazin A, reduced colonization by *V. cholerae* up to 100-fold at 100 and 200 μg doses. Further work with toxtazin A will be required to determine its mechanism of inhibition, and whether an effective concentration in vivo can be achieved that inhibits ToxT expression without inducing bactericidal activity.

3.3.2. *V. cholerae*: Inhibitors of Toxin Assembly. In a strategy primarily employed to inhibit the structurally related AB_5 toxin from enterotoxigenic *E. coli* (ETEC), heat-labile enterotoxin (LT), Hovey and colleagues used a crystallographic approach to identify inhibitors of toxin assembly of LT and CT.[90] A pharmacophore of the B pentamer pore was designed and used to computationally screen 158 758 molecules to identify compounds that blocked the pore, thereby preventing assembly of the A and B subunits. Due to the hydrophobicity of the pore, solubility was used to stratify initial hits, leading to the identification and cocrystallization with a lead hit, 3-(methylthio)-1,4-diphenyl-1*H*-1,2,4-triazolium bromide (MDT) (Figure 3). While binding affinity of MDT for the B pentamer was not reported and therefore would require significant follow-up before being considered as a therapeutic candidate, antiassembly inhibitors are an intriguing class of antivirulence agents that could serve as inhibitors for multiple toxins of the same structural family.

3.3.3. *V. cholerae*: Host Cell Receptor Mimics. Exerting their mechanism of action slightly downstream of toxin assembly, receptor mimics inhibit the assembled toxin from binding to host receptors. Fan et al. designed compounds that mimic the natural receptor of the CT B_5-subunit in humans.[91] This receptor, GM1 (Gal(β1−3)GalNAc(β1−4)[NeuAc(α2−3)]Gal(β1−4)Glc(β1−*O*-ceramide), is an oligosaccharide linked to a ceramide lipid that bears a terminal galactose residue. Using this galactose residue as a core element of their compounds, Merritt and colleagues used a structural approach to identify *m*-nitrophenyl-α-D-galactoside (MNPG) as an inhibitor of the related toxin LT of ETEC, which was later found by the same group to have an in vitro IC_{50} against CT of 720 μM by ELISA and to complex with CT in cocrystallography experiments (Figure 3).[91−93] Follow-up studies using focused screening libraries to optimize an inhibitor based on the MNPG scaffold identified a 2-thiophene acetyl derivative with modestly improved potency (see Gal derivatives, Figure 3).[94,95] Additional strategies to improve affinity included the synthesis of bifunctional (CTL 47)[96] and heterobifunctional ligands,[97] as well as pseudosugar mimics such as dicarboxy cyclohexanediol (DCCHD) (Figure 3).[98] The most successful derivatization of substrate mimics was reported by Fan et al. and Zhang et al. with the synthesis of multivalent ligands (see decavalent Gal, Figure 3).[99,100] Branched multivalent ligands with 5-fold symmetry achieved nanomolar binding affinities and were more potent than their nonbranched counterparts. Structural studies revealed that each branched multivalent inhibitor bound two B pentamers, with the inhibitor "sandwiched" between them.[99] Receptor mimics of GM1 are an attractive option for development as they could be used for both cholera and ETEC; however, many contain labile *O*-glycosidic linkages and can be highly insoluble, requiring further optimization before advancing as drug candidates.

3.3.4. *V. cholerae*: Inhibitors of Toxin Internalization. In a study primarily aimed at inhibiting internalization of Shiga

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toxin, Saenz and colleagues performed a cell-based HTS, identifying inhibitors of Stx1 internalization and two small-molecule inhibitors of CT function.[101] A benzodiazepine-derived molecule (compound **134**) was found to inhibit the toxin early in retrograde transport, when the toxin is transported to recycling endosomes (Figure 1). A compound with a scaffold bearing a triazole-imidazole heterobicyclic core (compound **75**) functioned later in the retrograde pathway, when the toxin is transported from recycling endosomes to the Golgi apparatus (Figure 1). These compounds were also found to inhibit Shiga-like toxins and diphtheria toxin, indicating their potential as antivirulence agents for multiple pathogens. However, it is likely that their targets are host proteins important for these trafficking functions.

3.3.5. *V. cholerae*: Inhibitors of ADP Ribosyltransferase Activity. In addition to targeting the assembly and receptor binding functions of CT, some inhibitors target the catalytic NAD^+-dependent ADP-ribosylation reaction. Zhang et al. designed and synthesized bisubstrate analogues based on NAD^+ and the ADP-ribose acceptor of $G_{\alpha s}$, arginine, to inhibit CTA1.[102] In a high-performance liquid chromatography (HPLC) based assay, the most potent compound, *N*-(4-bromobenzyl)-*N*′-(3-aminocarbonylbenzyl)guanidine (5q) had an IC_{50} of 31 μM, a 1400-fold increase in potency over NAD^+ (Figure 3). These preliminary findings can potentially be combined with available structural information to optimize inhibitors of CTA1.[83]

3.3.6. *V. cholerae*: Natural Product Inhibitors of Toxin Function. As an alternative strategy to high-throughput screening to identify antivirulence agents for *V. cholerae*, Zahid and colleagues characterized natural remedies that have been used for centuries to fight cholera infection.[88] Cholera is hypothesized to have originated in the Ganges delta region of south Asia and has been written about since the first recording of history in the region.[81] As such, peoples of the Indian subcontinent have developed many natural remedies for cholera over time, such as extracts of the plant *Berberis aristata*, red chili, sweet fennel, star anise seed, and others[88,103−105] and reviewed in ref 106. Oi and colleagues identified the active ingredient in the traditional Chinese medicine Daio-kanzo-to responsible for inhibiting CT activity.[105] Chemical extraction and fractionation of the original remedy identified rhubarb galloyl (RG)-tannin as the most potent inhibitor of CT-catalyzed ADP-ribosylation activity, which also protected against fluid accumulation in a dose-dependent manner in a rabbit ileal loop model (Figure 3). Synthesis of gallate derivatives, though less effective than RG-tannin, demonstrated that an increasing number of galloyl groups connected by depside bonds increased the potency of the compounds against CT. While a mechanism of action for these inhibitors has not been reported, we speculate that multigalloyl tannins function by interfering with the binding of CT to GM1 gangliosides.

Other studies have identified the protective effects of polyphenols. Saito and colleagues found apple polyphenol extract (APE) to inhibit ADP-ribosylation activity of CTA1 in a dose-dependent manner, with an IC_{50} of 8.7 μg/mL in vitro.[107] Crude APE partially protected against fluid accumulation in a mouse intestinal loop assay and a sealed mouse model. Fractionation of the crude APE showed that oligomeric and polymeric polyphenols were responsible for the protective effect against CTA1. One potential drawback of polyphenols is the reported vibriocidal properties of tea polyphenols;[108] thus, detailed pharmacokinetic studies would need to be carried out to ensure that the in vivo concentration in the small intestine was sufficient to inhibit CT activity, but not high enough to exhibit bactericidal effects, which could lead to increased selective pressure to develop resistance. Maintaining such a therapeutic window could prove challenging, especially in resource-poor areas in which cholera outbreaks occur.

A group led by Yamasaki has studied many natural remedies to characterize the constituent compounds responsible for inhibition of *V. cholerae*.[88,104,106] In two similar studies of red chili, the hydrophobic fraction containing capsaicin (*N*-vanillyl-8-methyl-nonenamide) was found to inhibit the production of CT, hypothesized to be due to transcriptional activation of histone-like nucleoid structuring protein (H-NS), which in turn downregulates transcription of ToxT (Figure 3).[104,106] However, it was reported that capsaicin failed to show in vivo efficacy.[88] Another study by the same group identified the compound *E*-anethole (*trans*-1-methoxy 4-propenyl benzene), derived from sweet fennel and star anise seeds (Figure 3).[88] In the rabbit ileal loop model, *E*-anethole decreased fluid accumulation and CT production in a dose-dependent manner. While *E*-anethole showed efficacy in this in vivo model, a potential drawback of both capsaicin and *E*-anethole is that both compounds exhibit bactericidal properties at higher concentrations.[88,104] Indeed, in the rabbit ileal loop model, doses of *E*-anethole required to decrease fluid accumulation to undetectable levels also reduced bacterial counts 1−2 orders of magnitude, as compared to untreated controls. The authors reported that this decrease in bacterial counts was not statistically significant, and some decrease in counts was to be expected given the coregulation of CT with TCP, where TCP is important for colonization. However, given the importance of TCP for colonization and survival of the bacterium, it raises the question of whether ToxT is the ideal target for an antivirulence agent, or if decreased ability to colonize the small intestine in the presence of drug will quickly select for resistant bacteria. Illustrating this point, a single point mutation in ToxT, L113P, was found to confer resistance to virstatin.[86]

3.3.7. *V. cholerae*: Inhibitors of the Cysteine Protease Domain. Another target of *V. cholerae* virulence is the multifunctional-autoprocessing-repeats-in-toxin (MARTX) toxin. Discovered in *V. cholerae* in 1999, it is less well characterized than CT, but has been found in nearly all clinical isolates of *V. cholerae* and contributes to virulence, cytotoxicity, and colonization[109−112] and reviewed in refs 113 and 114. MARTX toxin is a 4545 amino acid protein consisting of two domains of conserved MARTX sequences of glycine-rich repeats, an actin cross-linking domain (ACD), a Rho-inactivating domain (RID), an α/β hydrolase domain (ABH), and a cysteine protease domain (CPD).[113−115] The CPD is activated in the same manner as the CPD of TcdA and TcdB of *C. difficile*, where the CPD is allosterically activated by the mammalian-specific sugar 1D-*myo*-inositol hexakisphosphate (IP_6) to cleave off the two effector domains of the toxin.[116] The two effector domains of the toxin disrupt the host actin cytoskeleton: the ACD covalently cross-links actin monomers and the RID inhibits Rho GTPases.[113−115] Since cholera only causes minimal intestinal pathology, the role of these domains in vivo is less clear, but mutants lacking both the MARTX toxin and another accessory toxin, the cytolysin/hemolysin HlyA, are deficient at colonizing mouse intestines.[110] Current evidence best supports a role of the MARTX toxin and HlyA in evasion of the host immune system.[114] The first small-molecule inhibitors of MARTX toxin targeted the CPD, which activates

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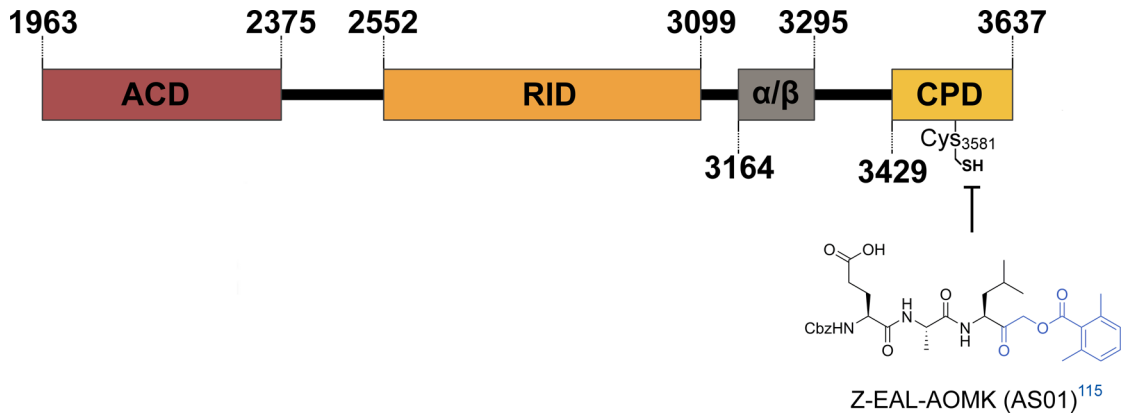

Figure 4. MARTX toxin of *V. cholerae* is made up of four primary functional domains, with cleavable loops shown in black. The flanking transmembrane repeats are not shown. Upon endocytosis, the cysteine protease domain (CPD; yellow) cleaves multiple loops in the polyprotein to activate toxic fragments consisting of the actin cross-linking domain (ACD; red) and the Rho-inactivating domain (RID; orange). The peptide acyloxymethyl ketone (AOMK) AS01 was designed based on the established cleavage sites for the CPD. It acts as a covalent irreversible inhibitor of the CPD that modifies the active site cysteine residue through the AOMK warhead (blue).

the toxin via proteolytic cleavage.[115] Using a focused library of 498 small molecules targeting cysteine proteases, Shen and colleagues screened for inhibitors of CPD-mediated autoprocessing of a pro-enzyme construct of the MARTX CPD. Of the eight initial aza-peptide epoxide hits, all contained a leucine in the P1 position, indicating substrate specificity of the CPD. Further SAR analysis revealed that increasing peptide length from a dipeptide to a tripeptide and *S,S* stereochemistry of the epoxide improved inhibitor potency. Finally, switching to an acyloxymethyl ketone (AOMK) electrophile known to target cysteine proteases over the epoxide warhead led to the most potent inhibitor, Z-EAL-AOMK (AS01) with a nanomolar AC_{50} (Figure 4). AS01 inhibited autoprocessing of MARTX toxin in *V. cholerae* cultures in a dose-dependent manner, but did not completely protect against autoprocessing at concentrations up to 100 μM. However, AS01 completely blocked the actin cross-linking function of the ACD at 50 μM when host cells were challenged with *V. cholerae* supernatants pretreated with AS01. One drawback of AS01 as a potential therapeutic is its negative charge, which likely affects cell permeability. This was seen when AS01 was added to the host cell media concurrently with wild-type *V. cholerae* supernatant, as there was significantly less protection against host cell actin cross-linking. Therefore, further SAR analysis to develop a cell-permeant inhibitor is required.

The antivirulence agents to treat cholera are interesting because many function upstream of toxin action, targeting transcriptional regulators of the toxin. While this strategy has the benefit of exerting its effects in the bacterium itself, because ToxT regulates the transcription of many genes, there may be higher selective pressure to develop resistance against inhibitors. Indeed, a ToxT mutant resistant to the small-molecule inhibitor virstatin was readily isolated.[86] Also, a significant number of antivirulence agents were derived from traditional medicines. As a majority of these compounds are derived from edible sources, these compounds could be used as prophylactic agents in areas of endemic disease. While significant research has been undertaken to develop these compounds, very few target the toxic ADP-ribosylation activity of CTA1. Due to the significant structural data on CTA1, as well as other similar ADP-ribosyltransferase toxins such as LT of enterotoxigenic *E. coli*, the development of drug-like small-molecule inhibitors of ADP-ribosylating toxins remains a significant opportunity.

3.4. Enterotoxigenic *Escherichia coli*

Strains of *E. coli* that express the virulence factors heat-labile enterotoxin (LT) and heat-stabile enterotoxin (ST) are termed enterotoxigenic *E. coli* (ETEC). ETEC is a major cause of diarrhea among children in the developing world as well as traveler's diarrhea.[117] The bacterium is transmitted through contaminated food and water and therefore tends to be endemic in locations without proper water infrastructure.[117] The characteristic symptom of ETEC infection is watery diarrhea, and the disease is usually self-limiting, although some patients develop extreme diarrhea and dehydration similar to infection with *V. cholerae*. The virulence factor LT elaborated from ETEC also shares significant similarity with cholera toxin (CT) of *V. cholerae*, with the toxins sharing 82% sequence identity.[82] Like CT, LT is structurally classified as an AB_5 toxin in which the fully assembled toxin contains one active "A" domain and five binding "B" domains. The A subunit functions as an ADP-ribosylating enzyme, adding an ADP-ribose group to the $G_{\alpha s}$ subunit, thereby leading to constitutive activation of $G_{\alpha s}$, stimulation of adenylyl cyclase, and pathologic increases in cAMP.

3.4.1. Enterotoxigenic *E. coli*: Inhibitors of Toxin Assembly. Using a structure-based approach, Hovey and colleagues identified inhibitors of LT assembly.[90] Based on the observation that the A subunit cannot assemble with a completed B pentamer,[118] molecules that blocked the entry point of the pore were identified to interfere with A–B assembly. A computational screen of 158 758 compounds was carried out to identify candidate inhibitors. The lead hit, 3-(methylthio)-1,4-diphenyl-1*H*-1,2,4-triazolium bromide (MDT), was chosen for follow-up primarily due to its solubility (Figure 3). Structural studies of the complex between MDT and LT showed the compound binding in three different locations along a single pentamer. However, the binding affinity of MDT for the B pentamer was not reported and therefore would require significant follow-up before being considered as a therapeutic candidate. Additionally, due to the inherent hydrophobicity of the B subunit pore, solubility of drug leads is likely to be a significant hurdle. However, this compound likely inhibits B pentamers of other bacterial toxins with high

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sequence identity, such as CT, making antiassembly inhibitors an intriguing class of antivirulence agents that could serve as inhibitors for multiple toxins of the same structural family.

3.4.2. Enterotoxigenic *E. coli*: Host Cell Receptor Mimics. Another strategy to target LT is to develop mimics of the receptor binding motif of the B pentamer. Merritt and colleagues used a structural approach to identify inhibitors based on the natural receptor binding site, GM1 (Gal(β1−3)GalNAc(β1−4)[NeuAc(α2−3)]Gal(β1−4)Glc(β1−*O*-ceramide), an oligosaccharide linked to a ceramide lipid that bears a terminal galactose residue.[92] By cocrystallizing derivatives of the GM1 terminal saccharide galactose with LT, *m*-nitrophenyl-α-D-galactoside (MNPG) was identified as the most potent inhibitor, albeit with a modest potency of 500 μM (Figure 3). Further work confirmed that MNPG also inhibited the B pentamer of CT with an in vitro IC_{50} against CT of 720 μM[93] and identified derivatives of MNPG with inhibitory properties (Figure 3).[91,94,95] These studies showed that monomers of receptor mimics were not viable leads due to their lack of potency. To improve upon the potency of monomeric receptor mimics, Fan et al. (for LT) and Zhang et al. (for CT) designed multivalent ligands with 5-fold symmetry that achieved nanomolar binding affinities.[99,100] Receptor mimics of GM1 are an attractive option for development as they could be used for both cholera and ETEC; however, many contain labile *O*-glycosidic linkages and can be highly insoluble, requiring further optimization before advancing as drug candidates. In addition to the inhibitors highlighted above, it is possible that small-molecule inhibitors designed for CT could be used as scaffolds for the development of LT inhibitors.

3.5. *Cornybacterium diphtheriae*

Diphtheria is an upper respiratory tract infection caused by the bacterium *C. diphtheriae*.[119,120] Symptoms include sore throat, fever, and swollen lymph nodes, with a characteristic gray pseudomembrane formation on the back of the throat.[119,120] Cardiac and neuronal involvement occurs in severe cases.[119,120] Due to the highly successful diphtheria vaccine, this infection is rare in the United States, but is still prevalent in developing countries around the world without access to the vaccine. Morbidity and mortality of infection are largely mediated by the virulence factor diphtheria toxin (DT).[119,120] DT is synthesized as a single polypeptide (567 amino acids) composed of a catalytic A domain, a translocation domain, and a receptor binding domain.[120] The translocation and receptor binding domains make up the B subunit of this toxin, while the A subunit has ADP-ribosyltransferase activity and catalyzes the ADP-ribosylation of eukaryotic elongation factor 2 (eEF2), halting protein synthesis and leading to cell death.

3.5.1. *C. diphtheriae*: General Antitoxin Strategies. While DT is a major virulence factor for diphtheria, few antivirulence strategies have been developed. One study by Zhou and colleagues designed transition state mimics of NAD^+ as inhibitors of the A subunit of DT (DTA), pertussis toxin (PTA), and cholera toxin (CTA).[121] Two compounds inhibited ADP-ribosylation with K_i values in the micromolar range, but these values were similar to the K_i of the NAD^+ substrate, indicating that transition state analogues of PTA and DTA do not bind more tightly to the enzyme than the substrate. Additionally, a study primarily aimed at discovering inhibitors of lethal toxin (LT) of anthrax infection identified endosome acidification inhibitors that also disrupted the internalization process of DT.[122] Two compounds, the natural product diphyllin and the clinically used antihelmithic niclosamide, completely protected cells from DT challenge at 5 μM (Figure 1).

Significant efforts to develop antivirulence agents have not been undertaken for DT, possibly due to the readily available and highly successful vaccine that has largely eliminated infection in developed countries. However, the WHO estimates there are almost 5000 annual cases worldwide with a 5−10% mortality rate and many more cases are likely to go unreported.[123,124] This remaining disease burden justifies efforts to make the vaccine more widely accessible worldwide, as well as to further develop antivirulence agents against DT.

3.6. *Pseudomonas aeruginosa*

Infection with *P. aeruginosa* is typically hospital-acquired in patients with underlying medical conditions or compromised immune systems.[125] Listed as a serious health threat due to multidrug resistance (Table 1), infections with *P. aeruginosa* cause pneumonia, urinary tract infections, septicemia, and surgical site infections.[6,125] Particularly notable is its propensity to cause pneumonia in patients with cystic fibrosis and those on ventilators.[125] Of the approximately 50 000 cases of *P. aeruginosa* infections per year in the United States, 13% or 6700 are multidrug resistant, and some strains of this bacterium have been found to be resistant to all or nearly all antibiotics.[6] Therefore, novel treatments for this infection represent a critical unmet need.

3.6.1. *P. aeruginosa*: Inhibitors of ADP Ribosyltransferase Activity. *P. aeruginosa* elaborates multiple virulence factors, including the toxin exotoxin A (ETA) (638 amino acids) that catalyzes the ADP-ribosylation of eukaryotic elongation factor 2 (eEF2). ETA is synthesized as a single polypeptide with an N-terminal receptor binding domain, a translocation domain, and a C-terminal ADP-ribosylstransferase domain.[125] The toxin is cleaved by furin on the cell surface into its A and B subunits, which are linked by a disulfide bond. Upon receptor-mediated endocytosis and retrograde transport, reduction of the disulfide bond and release into the cytosol allows the A subunit to ADP-ribosylate and inactivate eEF2, halting protein synthesis and causing cell death. Based on similarity to other ADP-ribosylating enzymes, substrate mimics identified as inhibitors of other enzymes in this functional class have been tested against ETA in vitro. Armstrong and colleagues screened a small set of NAD^+ analogues for inhibitory activity against ETA.[126] Compounds were derived based on a parent molecule, 1,8-naphthalimide (NAP), a known inhibitor of other ADP-ribosyltransferase enzymes, including the eukaryotic poly (ADP-ribose) polymerase enzymes (PARPs) (Figure 5A). Despite attempts at optimization, NAP was the most potent compound in vitro with an IC_{50} of 87 nM. While NAP displayed good potency toward ETA, the compound had poor solubility, leading the group to further optimize inhibitors for hydrophilicity. Yates et al. screened a focused set of NAD^+ analogues with a benzamido group fused to a functionalized hetero-ring structure.[127] These compounds were water-soluble and displayed similar potency as NAP, with the most potent compounds having IC_{50} values in the nanomolar range. A cocrystal structure was solved with one of the top inhibitors, *N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-2-(4-pyrrolidin-1-yl)acetamide-HCl (PJ34), a compound previously characterized as a PARP inhibitor (Figure 5A). While NAP and PJ34 have good potency toward ETA, because

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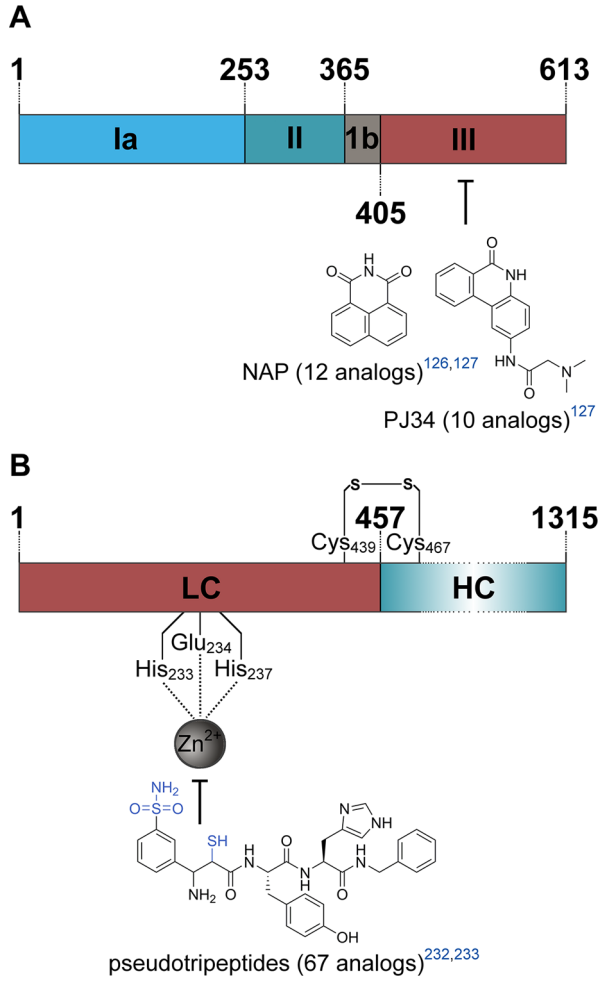

Figure 5. Compounds targeting major virulence factors of *P. aeruginosa* and *C. tetani*. (A) *P. aeruginosa* produces exotoxin A (ETA) consisting of a receptor binding domain (Ia), a translocation domain (II), a small domain with an unknown function (Ib) and an ADP-ribosylating domain (III). Multiple lactam-based compounds target the ADP-ribosylating domain. (B) *C. tetani* tetanus toxin (TeNT) is composed of a zinc-dependent metalloprotease (LC; red) and a heavy chain (blue) linked by a disulfide bond. A small library of pseudotripeptides bind to the zinc ion, with the reactive sulfur atom and sulfonamide that chelate the metal ion shown in blue.

they were both previously identified as inhibitors of eukaryotic enzymes, cross-reactivity with the host may lead to toxicity.

3.7. *Bordetella pertussis*

Whooping cough is a bacterial infection caused by the gram-negative bacterium *B. pertussis*. This upper respiratory infection has been largely eradicated by vaccines in the developed world, but still caused almost 140 000 infections worldwide in 2014 with an estimated 89 000 deaths.[128] Several virulence factors mediate pathology, including pertussis toxin (PT) and the adenylyl cyclase toxin CyaA.[129] PT is structurally classified as an AB_5 toxin, where the A subunit has ADP-ribosyltransferase activity. Despite its similarity to other toxins with ADP-ribosyltransferase activity, such as cholera toxin (CT) and diphtheria toxin (DT), no viable antivirulence agents have been developed for this toxin. Potent inhibitors of CTA may serve as scaffolds for development.

3.7.1. *B. pertussis*: Inhibitors of Adenylyl Cyclase Activity. The adenylyl cyclase CyaA is important in early stage colonization and contributes to the lethality of infection.[129] It shares homology with edema factor (EF) from *Bacillus anthracis* and ExoY from *P. aeruginosa*. Similar to PT, few studies have been carried out to design inhibitors of CyaA. One study performed by Soelaiman and colleagues primarily aimed at identifying EF inhibitors also tested these compounds against CyaA.[130] Using the structure of EF, 205 226 small molecules were docked into the 3′-dATP binding site to find inhibitors. Two of the top quinazoline-based hits were screened against the N-terminal adenylyl cyclase domain of CyaA in vitro and had IC_{50} values of 40 and 80 μM against CyaA and EF, respectively, making them more potent inhibitors of CyaA than EF (see 277890, Figure 6). This study showed that non-nucleoside-based inhibitors of adenylyl cyclase toxins could inhibit multiple toxins with adenylyl cyclase activity; however, potency must be improved before quinazolines can be tested in vivo.

3.8. *Bacillus anthracis*

B. anthracis is a gram-positive spore-forming bacterium and the causative agent of anthrax.[131] Human infection is infrequent and usually caused by exposure to infected animals or animal products.[131] Cutaneous infection is the most common type of infection and occurs when contaminated animal products are handled. This form is usually easily treatable.[131] A recent example of an anthrax outbreak believed to be caused by infected reindeer occurred in July and August of 2016, where thawing of the permafrost in Siberia uncovered 75-year-old infected reindeer carcasses.[132,133] Subsequent infection of the reindeer population in the Yamal peninsula of Russia spread to nomadic populations, resulting in one death out of eight confirmed cases.[133] While outbreaks are rare, the potential to use anthrax as a weapon of bioterrorism is significant, as was demonstrated in the United States in 2001 when bacterial spores were mailed to media and government locations resulting in five fatalities from 22 confirmed anthrax cases.[131,134] In inhalational anthrax, the most deadly and most likely route of dissemination in the event of a bioterrorist attack, initial symptoms are flulike and difficult to differentiate from other more benign causes of upper respiratory infections.[131] Fulminant disease occurs within 1−3 days with death closely following.[131] Standard of care treatment is antibiotics; however, they must be initiated early for success.[131] Given the generic nature of the initial symptoms, early diagnosis and timely treatment is a significant challenge.

Pathogenicity of *B. anthracis* is mediated by three virulence factors: the capsule and two exotoxins.[131] The virulence factor that most contributes to the deadly symptoms seen in infection is a three-protein toxin comprised of protective antigen (PA), lethal factor (LF), and edema factor (EF).[135] Interestingly, none of these toxins are virulent on their own—PA must combine either with LF to form lethal toxin (LT, LeT, or LeTx) or with EF to form edema toxin (ET).[131,135,136] LT is largely considered to be the primary virulence factor.[135] PA is an 83 kDa protein that acts as a pore to translocate LF or EF into the cytosol.[135] PA is first cleaved by host proteases such as furin in the extracellular space, where the 63 kDa fragment oligomerizes into a heptamer or octamer.[135] Upon binding LF or EF in the extracellular space, LT or ET is internalized by endocytosis.[135] A decrease in the pH of the endosomal compartment allows PA to insert into the membrane and mediate translocation of LF or EF into the cytosol of host cells.[135] EF acts as a calmodulin-dependent adenylate cyclase

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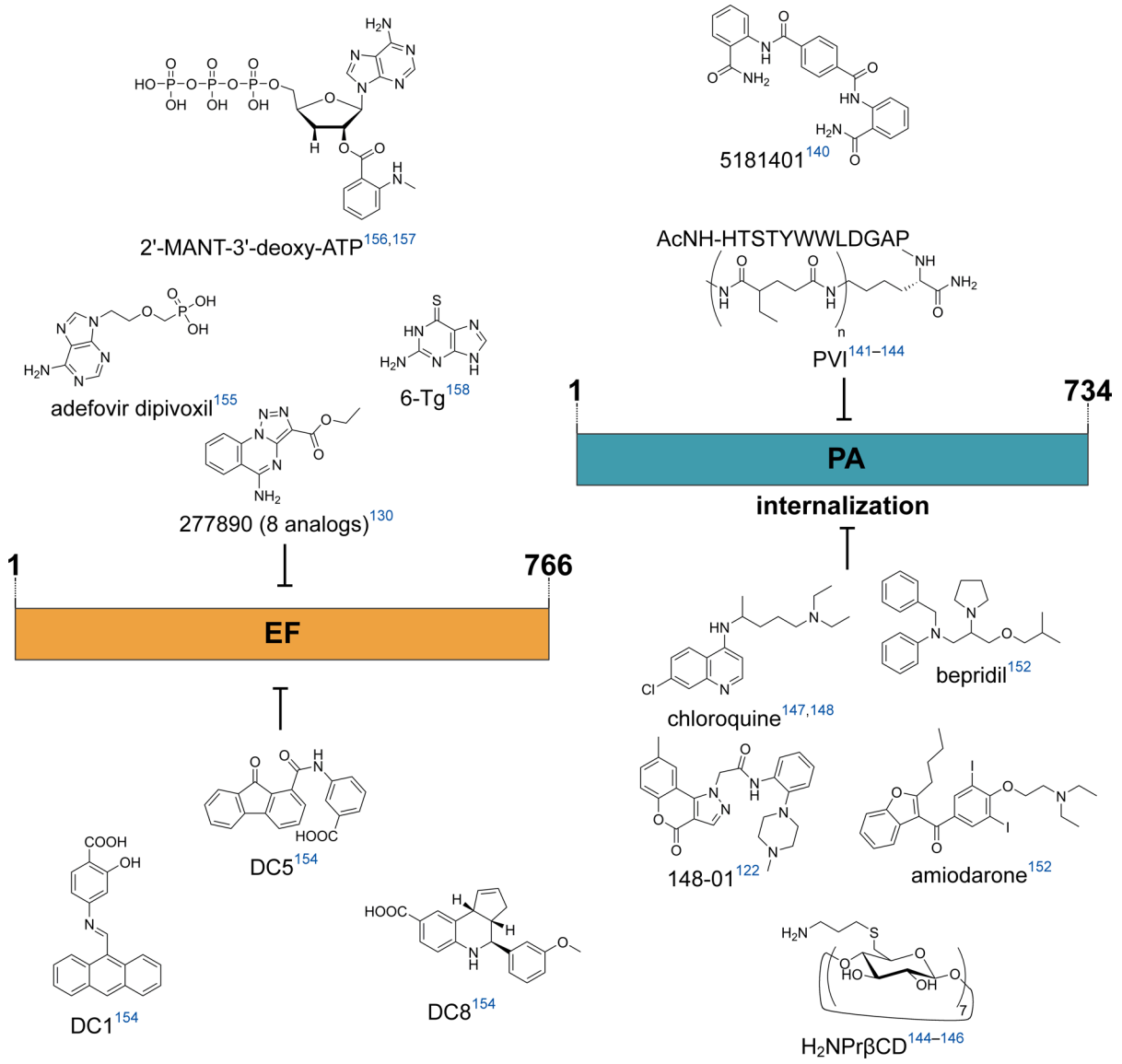

Figure 6. Compounds that target edema factor (EF) or protective antigen (PA) of *B. anthracis*. EF is shown in yellow and PA is shown in blue. To form a functional toxin, either EF or lethal factor (see Figure 7) assembles with PA. Since both toxins need an activated PA, many compounds target the activation and assembly of PA (upper right) or its binding to the host receptor and the internalization process (lower right). Substrate mimics of EF that target its adenylyl cyclase activity are shown on the left.

and increases the second messenger cAMP to pathologically high levels, leading to edema and vascular dysfunction. LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKKs or MEKs) and NLRP1 inflammasome sensors (in rodents).[135]

Significant efforts have been undertaken by academic and industry scientists to develop therapies for anthrax. Current standard of care treatments consist of antibiotics to kill the bacterium; however, antibiotics become ineffective later in the course of infection when large amounts of toxin have already been secreted.[131] Antibiotics are similarly ineffective against anthrax spores.[131] Further, the greatest threat of disseminated anthrax infection is likely in the form of a weapon of bioterrorism. In this case, strains will likely be selected or engineered to be resistant to many antibiotics, strengthening the rationale for the development of antitoxin strategies to treat infection. An ideal therapy for anthrax could be made in large quantities and stored for long periods of time, easily distributed and taken by at-risk populations, and could be used prophylactically or in combination therapy with antibiotics.

Since PA is required for pathogenicity of both LF and EF, it has been the most significant target for antitoxin strategies. The licensed vaccine Anthrax Vaccine Adsorbed (AVA, or BioThrax) induces antibodies against PA_{83}.[137] Additionally, the FDA-approved human monoclonal antibody raxibacumab and the recently approved obiltoxaximab (approved in March 2016) both target PA.[138,139]

3.8.1. *B. anthracis*: Inhibitors of Toxin Assembly. One strategy to target PA is to inhibit its oligomerization. Wein et al. performed a virtual screen of 10 000 compounds to identify PA binders that inhibit oligomerization.[140] Through in vitro secondary screening, the lead compound bis[2-(aminocarbonyl)phenyl]-1,4-benzenedicarboxamide (5181401) was identified with an EC_{50} in the low micromolar range in cell-based assays (Figure 6). The compound not only inhibited PA oligomerization, but also prevented cleavage by

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the host cell protease furin. Molecules that inhibit PA oligomerization are attractive due to their ability to prevent both LT- and ET-mediated cell pathology and because they do not need to be cell-permeant. However, before becoming a drug lead, this compound needs further optimization to improve solubility and potency.

Peptide-based approaches have also been used to inhibit PA. Using phage display, Mourez and colleagues identified two peptide sequences that bound to PA_{63} near the binding site of LF and EF.[141] These two peptide sequences, HTSTYWWLDGAP (P1) and HQLPQYWWLSPG (P2), both contained the hydrophobic sequence YWWL, which the authors hypothesized was important for binding to PA_{63} (Figure 6). Due to the weak inhibitory potency of P1 with an IC_{50} of ~150 μM, a polyvalent inhibitor covalently bound to polyacrylamide was synthesized. Each polyvalent inhibitor (PVI) molecule contained an average of 22 P1 peptides and ~900 acrylamide monomers (Figure 6). Polyvalency of P1 improved the IC_{50} to 20 nM (per molar concentration of linked peptide). In a rat intoxication model of PA and LF in which rats were challenged with 10 times the minimal lethal dose of PA and LF, a low dose of PVI delayed symptoms when compared to control, and high doses abolished symptoms completely. Further, PVI was protective when injected 3−4 min after challenge, indicating that, as a therapy, it may be effective after infection. Similar studies using phage display to design inhibitors of PA_{63} at the LF binding site confirmed the importance of YWWL hydrophobic residues in consensus sequences.[142,143] Using competitive elution of peptides from PA_{63} with LF to identify PA_{63} inhibitors at the LF binding site, the optimized 12-mer NAMTYWWLDPPL was synthesized into a tetra-branched multiple antigen peptide (MAP) with a trilysine core (MAP3 V/A).[142] MAP3 V/A had an EC_{50} of 65 nM in a cell viability assay with LF, inhibited EF-induced increases in cAMP levels in cells, and a 1 mg injection of MAP3 V/A completely protected rats in an in vivo intoxication model (11/11) from LF-induced mortality. These studies highlight the potential improvement in potency with multivalency for targeting large, multisubunit complexes.

Using the 7-fold symmetry of oligomerized PA, Karginov et al. synthesized β-cyclodextrin derivatives to block the PA pore.[144,145] Modification to include positive charges to interact with the negatively charged PA pore yielded a low molecular weight compound, per-6-(3-aminopropylthio)-β-cyclodextrin ($H_2NPr\beta CD$), that blocked ion conductance of PA with an IC_{50} of 0.55 nM (Figure 6). In a rat intoxication model, high doses of $H_2NPr\beta CD$ given as a pretreatment or concurrently with toxin completely protected rats from toxin-induced death. Follow-up studies explored hepta-6-alkylarylamine β-cyclodextrin derivatives with nanomolar IC_{50} values in vitro (Figure 6).[146] Due to the potency, low molecular weight, and solubility of cyclodextrins, these compounds are interesting candidates for further study.

3.8.2. *B. anthracis*: Inhibitors of Toxin Internalization. Many compounds have been identified that prevent proper internalization of the toxin. The known antimalarials chloroquine (*RS*)-*N*′-(7-chloroquinolin-4-yl)-*N*,*N*-diethyl-pentane-1,4-diamine) and quinacrine ((*RS*)-6-chloro-9-(4-diethylamino-1- methylbutylamino)-2-methoxyacridin), as well as other lysosomotropic amines, were studied for their ability to block endosomal acidification required for toxin internalization (Figures 1 and 6). Using NH_4Cl and chloroquine, Friedlander blocked LT-induced macrophage cytotoxicity, showing the requirement of endosomal acidification for proper LT transport into the cell.[147] In animal intoxication models, chloroquine partially protected mice from death (15/48) compared to controls (1/17), using dosing regimens similar to those used to treat humans with malaria, showing partial efficacy at therapeutically relevant concentrations.[148] Quinacrine was originally used as a small-molecule tool to uncover the mechanism of LT-induced cytotoxicity through its proposed function as a phospholipase A_2 inhibitor.[149] At 20 μM, quinacrine blocked 97% of LT-induced cytotoxicity in macrophages, leading the authors to conclude that cytolysis by LT is mediated through phospholipase A_2 activity. Follow-up studies showed that quinacrine also inhibited ET-induced increases in cAMP, leading the authors to conclude that it is likely the dibasic properties of quinacrine increasing endosomal pH that accounts for its protective effects against both LT and ET.[150] However, in mouse and guinea pig animal models, quinacrine offered no protection up to doses that were toxic to the animals. While quinacrine was ineffective in vivo, the efficacy of the approved drug chloroquine at therapeutically relevant concentrations indicates its potential to be repurposed for use in anthrax infection.

Zhu and colleagues performed a quantitative high-throughput screen (qHTS) of 70 094 compounds to identify small-molecule inhibitors of toxin internalization using Förster resonance energy transfer (FRET) to detect internalization of a nontoxic LF-β-lactamase (LF-β-lac) fusion protein.[122,151] Of 1170 compounds that showed dose-dependent protection of FRET activity, 30 compounds were chosen for follow-up based on high confidence of their activity in the screen or known biological activity. Three compounds, the natural product diphyllin (Figure 1), the clinically used antihelmithic niclosamide (Figure 1), and the novel compound NCGC00084148-01 (148-01, chromeno[4,3-*c*]pyrazol-4(1*H*)-one) (Figure 6), protected cells from LF-mediated toxicity in the range of 3−10 μM. The most potent compound, diphyllin, also protected RAW264.7 macrophages when added 45 min after treatment with up to 1000 ng/mL LT. While these initial results showed that these three compounds inhibited LT, further studies revealed that all three compounds also protected against toxicity induced by the fusion protein FP59 containing the N-terminus of LF and the catalytic domain of *Pseudomonas* exotoxin A, as well as diphtheria toxin (DT). This indicated that the primary function of these compounds was most likely inhibition of the common internalization pathway used by these toxins. Indeed, the three compounds performed similarly to bafilomycin A1 (Figure 1), an inhibitor of vacuolar-type H^+-ATPase, in preventing PA from inserting into the endosomal membrane, classifying these compounds as inhibitors of endosome acidification.

A phenotypic cell-based screen was employed by Sanchez et al. to find compounds that inhibited toxin entry into host cells.[152] By screening ~500 biologically active small molecules for compounds that inhibited LT-mediated cell death in RAW 264.7 macrophages, two clinically used compounds that treat cardiac arrhythmias, amiodarone and bepridil (no longer used in the United States due to adverse drug effects), were found to protect cells against LT-mediated killing with IC_{50} values of 3.5 and 4.8 μM, respectively (Figure 6). Further analysis indicated that these compounds likely act to inhibit acidification of endosomes. However, these compounds are known to have other cellular effects including calcium channel blocking, the mechanism by which they act to treat arrhythmias.[153] Further,

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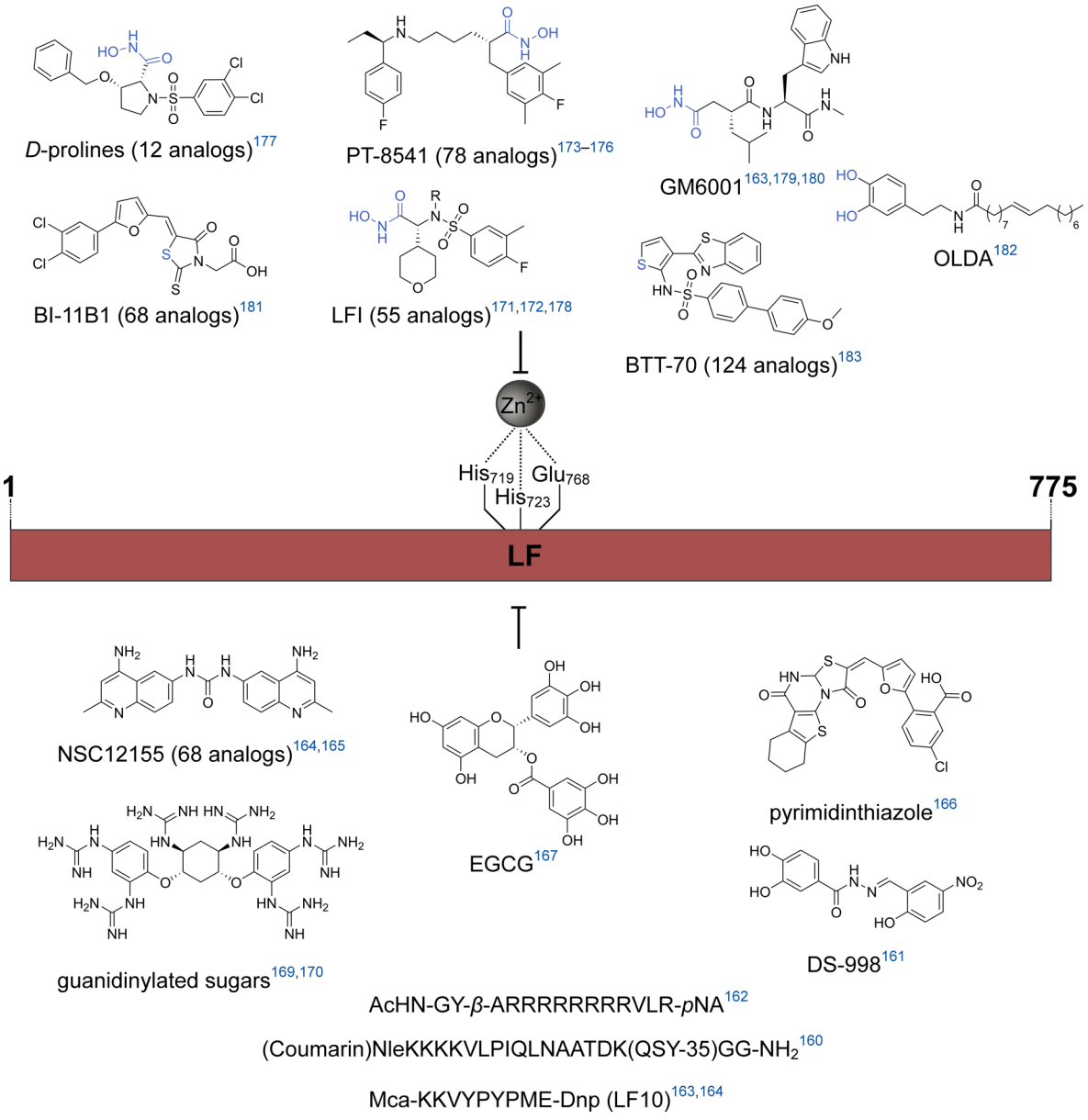

Figure 7. Compounds targeting lethal factor (LF) of *B. anthracis*. To assemble an active lethal toxin, LF (red) binds to PA (see Figure 6). Functionalized sugars as well as aromatic and peptide-based compounds target LF metalloprotease activity (lower). Upper compounds target the metal binding site (created by His_{719}, His_{723}, and Glu_{768}), with residues that chelate the zinc ion highlighted in blue.

amiodarone is known to have a variety of other cellular effects.[153] In a Fischer 344 rat intoxication model, a high dose of amiodarone (54 mg/kg) modestly delayed average time to death compared to controls (82.6 min versus 61.2 min); however, higher drug concentrations could not be achieved due to dose-limiting host toxicity, which also makes use of this compound as a drug lead problematic.

Slater and colleagues identified several compounds that protected against LT at distinct steps of toxin internalization and action using a phenotypic screen of LT-induced J774A.1 murine macrophage cell death.[76] Of the 31 350 small molecules screened, 49 compounds completely protected cells without cellular toxicity. The terpene toosendanin (Figure 1), a natural product from the plant *Melia azedarach* var. *toosendan* known to inhibit botulinum toxin in vivo,[77] as well as a glycosylated sterol natural product (5) belonging to the family of saponins, both prevented acidification of the endosome. Toosendanin had an IC_{50} of 56 nM against LT and protected cells at concentrations in which endosomal acidification was only minimally inhibited, indicating that it may act via multiple mechanisms. The possibility that toonsendanin acts via multiple mechanisms of action, together with its documented in vivo activity against botulinum toxin, could make it an attractive drug lead to follow up for use against multiple bacterial toxins. Interestingly, the authors also noted that many of the compounds identified in this screen also inhibited toxicity induced by toxin B of *C. difficile*. While many of the compounds that prevent toxin internalization have not been tested in in vivo models of anthrax, it is exciting that these small-molecule inhibitors inhibit multiple toxins. These studies identified one common pathway that could be targeted to develop an antivirulence agent to treat multiple bacterial infections.

3.8.3. *B. anthracis*: Inhibitors of Adenylyl Cyclase Activity. Another approach to block anthrax infection is to

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target the toxic effector proteins of the toxins: EF and LF. Using docking to screen compounds, Soelaiman et al. screened 205 226 small molecules to find compounds that bind in the 3′-dATP binding site of EF.[130] A total of 24 compounds were chosen based on the docking results or structural similarity to top hits and tested in vitro with EF. Seven of the top eight compounds contained a core quinazoline moiety and inhibited EF adenylyl cyclase activity with IC_{50} values from 25 to >1000 μM. Top hits were further characterized by promiscuity toward other enzymes and host cell adenylyl cyclase types I, II, and V. The lead hit, ethyl 5-amino[1,2,3]triazolo[1,5-*a*]quinazoline-3-carboxylate (277890) acts as a direct inhibitor of EF (Figure 6). It binds in the ATP binding site, did not inhibit the host adenylyl cyclase isoforms tested, and protected against EF-induced toxicity in cells when used at high micromolar concentrations. This compound was the first non-nucleoside-based inhibitor of adenylyl cyclase toxins and was found to also inhibit the adenylyl cyclase CyaA from *B. pertussis*, which shares 35% sequence identity with EF, making this compound a potential therapeutic lead for multiple toxins acting via adenylyl cyclase activity. However, the low potency of this compound in vitro and in situ suggests that significant medicinal chemistry efforts will be required before quinazolines could be tested in vivo. Computational screening and fragment-based design using the EF pharmacophore was used by Chen and colleagues to identify three non-nucleoside inhibitors, 3-[(oxo-9*H*-fluorene-1-carbonyl)amino]benzoic acid (DC5), 4-(3-methoxyphenyl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolone-8-carboxylic acid (DC8), and 4-[(anthracen-9-ylmethylene)amino]-2-hydroxybenzoic acid (DC1), with single digit micromolar IC_{50} values in vitro (Figure 6).[154] These molecules were selected based on their drug-like properties and diverse core scaffolds, making them interesting scaffolds for further optimization and in vivo testing.

To find other inhibitors of EF, Shen and colleagues tested clinically approved nucleotide analogues.[155] Prodrugs and metabolites of the acyclic nucleoside phosphonate adefovir dipivoxil (9-[2-[[bis[(pivaloyloxy)methoxy]phosphinyl]-methoxy]ethyl]adenine, or bis-POM-PMEA), approved for the treatment of chronic hepatitis B virus (HBV), inhibited EF in an in vitro adenylyl cyclase assay (Figure 6). The cellular metabolite PMEAapp, which contains a phosphate, competitively inhibited EF with a K_i of 27 nM, a 10 000-fold increase in potency over its affinity for ATP (K_m = 168−194 μM) and had at least 10-fold lower affinity for mammalian adenylyl cyclase types I, II, and V compared to EF. Adefovir dipivoxil protected cells from increased cAMP levels and ET-induced morphological changes with an IC_{50} of approximately 0.1 μM. PMEApp also competitively inhibited CyaA of *B. pertussis* with a K_i of 25 nM. In addition to being a clinically approved compound, the improved potency over the non-nucleoside quinazoline inhibitor makes adefovir a more promising candidate to move forward into in vivo studies. However, adefovir has a significantly reduced specificity compared to the non-nucleoside compound, which could lead to adverse effects in animal systems. Modification of purines and pyrimidines with *N*-methylanthraniloyl (MANT) led to the development of reversible and competitive inhibitors of EF with in vitro K_i values of 110 nM for MANT-CTP and 10 nM for 3′-MANT-2′-deoxy-ATP (Figure 6).[156,157] Due to their fluorescence, these compounds can also be used as probes to study EF or to develop high-throughput screens to identify novel EF inhibitors. While these MANT-substituted nucleotides are potent inhibitors, potential drawbacks include cross-reactivity with host cell adenylyl cyclases, and their impermeability to the plasma membrane due to charge. Additionally, some purine nucleosides are reported to inhibit spore germination, which may increase selective pressure to evolve bacteria with resistance to these compounds (see 6-Tg, Figure 6).[158]

3.8.4. *B. anthracis*: Inhibitors of the Metalloprotease Domain. The majority of inhibitors have been developed against LF, the main virulence factor of *B. anthracis*. In the first study to show zinc-dependent metalloprotease activity of LF, synthetic peptides were used to show the importance of proline in the P1 position and tyrosine in the P1′ or P2′ site.[159] Improving upon this substrate specificity, Cummings et al. compiled a consensus sequence based on analysis of MEK/MKK substrate sequences to design a quenched fluorogenic 16-mer peptide substrate for FRET assays.[160] The optimized sequence, (coumarin)NleKKKKVLPIQLNAATDK(QSY-35)GG-NH_2, with cleavage between the Pro-8−Ile-9 bond, was reported to have 100-fold increased turnover compared to the sequence of MEK1 and was effective in a plate reader assay to quickly screen for LF inhibitors (Figure 7). This optimized peptide sequence was used by Min et al. to design a mass spectrometry based approach to find LF inhibitors using self-assembled monolayers (SAMs) with matrix-assisted laser desorption ionization (MALDI) in a methodology they termed SAMDI.[161] The SAM consisted of the nonfluorogenic peptide from Cummings et al. (NleKKKKVLPIQLNAATDKGGC)[160] bound to a tri(ethylene glycol) backbone. Using this approach, 10 000 molecules were screened to find inhibitors of LF-mediated cleavage of the peptide. With a 0.1% overall hit rate, 11 compounds that blocked cleavage were identified, with one compound, 3,4-dihydroxy-*N*′-(2-hydroxy-5-nitrobenzylidene)-benzohydrazide (DS-998), noncompetitively inhibiting cleavage with a K_i of 1.1 μM in vitro (Figure 7). However, DS-998 exhibited cytotoxicity to cells in the J774 macrophage cell line at concentrations of 10 μM and above and only protected 60% of cells at 5 μM, indicating that this compound is likely not a viable candidate to move into animal models. Using a similar approach as Cummings and colleagues to find a consensus sequence from MEK substrates, Tonello and colleagues synthesized the peptide substrate AcHN-GY-β-ARRRRRRRRVLR-*p*NA (In-2-LF) for high-throughput screening of inhibitors (Figure 7).[162] The hydroxamate version of this peptide substrate was a potent inhibitor of LF activity, with a K_i of 1 nM measured in vitro and in a cell-based assay.

Using a mixture-based peptide library approach, Turk and colleagues determined LF substrate selectivity.[163] The P1′ position was the most selective, requiring hydrophobic residues such as tyrosine, while P2 required a basic residue. This unbiased approach yielded the quenched fluorescent LF10 peptide (Mca-KKVYPYPME-Dnp) with 50-fold more efficient cleavage by LF than a consensus sequence peptide based on MKK substrates (k_{cat}/K_M of 130 000 M^{-1} s^{-1} for LF10 compared to 2500 M^{-1} s^{-1} for the MKK1 sequence) (Figure 7). Interestingly, extending the peptide to a 15-mer (Mca-RRKKVYPYPME-Dnp-TIA, called LF15) further increased cleavage efficiency to a k_{cat}/K_M of 4×10^7 M^{-1} s^{-1}. Using these fluorogenic substrates, the peptide-based inhibitor MKARRKK-VYP-hydroxamate was found to have a K_i^{app} of 1.1 nM. The small-molecule inhibitor GM6001 (3-(*N*-hydroxycarboxamido)-2-isobutyl-propanoyl-Trp-methylamide) had a less potent K_i^{app} of 2.1 μM, but was efficacious in cell-based assays and protected cells from LF-mediated death when added up to 3 h

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after LT (Figure 7). In a parallel study, Panchal et al. used LF10 from the previous study[163] in a HTS of 1990 compounds to identify organic small-molecule inhibitors of LF activity.[164] Of the 19 compounds with >50% inhibition at 20 μM, two small organic molecules were chosen for follow-up based on their favorable chemical properties as potential therapies. Using these scaffolds, molecular modeling and three-dimensional database mining was carried out to identify a pharmacophore for LF binding. This led to the identification of a total of six LF inhibitors. The most potent inhibitor in vitro, *N*,*N*′-bis(4-amino-2-methyl-6-quinolinyl)-urea (NSC 12155) competitively inhibited LF with a K_i of 0.5 μM, but failed to completely protect cells from LT at 100 μM inhibitor concentration, likely due to poor cellular permeability (Figure 7). This highlights the importance of overall charge, solubility, and lipophilicity of the inhibitor in designing a potential drug lead. Using NSC 12155 as the starting compound, a medicinal chemistry study was performed by Williams et al., identifying more drug-like compounds with similar in vitro potency and improved selectivity over other metalloproteases such as BoNT A and human matrix metalloproteases (MMPs).[165] In a high-throughput screen of 10 000 drug-like molecules, Schepetkin et al. sought to identify LF inhibitors with distinct chemical structures from previously identified small-molecule inhibitors of LF.[166] These efforts resulted in four lead compounds possessing novel fragments such as 2-phenylfuran, *N*-phenyl-dihydropyrazole, *N*,*N*-diphenylurea-sulfonamide, and a carboxylic *N*-phenylpyrrole, with nanomolar K_i values in vitro (see pyrimidinthiazole, Figure 7). Interestingly, though these hits represented several diverse chemical core scaffolds, modeling showed similarities in docking to the pharmacophore published by Panchal et al.[164] Libraries containing these core moieties can be screened to further optimize LF inhibitors.

Using a natural products approach, Dell'Aica et al. tested extracts of green tea, which are known inhibitors of MMPs, against LF activity.[167] The most potent natural product, (−)-epigallocatechin-3-gallate (EGCG), inhibited LF in vitro with an IC_{50} of 97 nM (Figure 7). EGCG also protected cells against LF-mediated toxicity at concentrations as low as 10 nM. However, the protective effect of EGCG in a rat intoxication model was mixed, and the authors could not determine the mechanism by which EGCG inhibited LF, so further studies are necessary before this compound could be considered for development as a therapeutic lead. Based on this initial report, Numa and colleagues screened compounds with galloyl and 5-hydroxydopamine moieties.[168] The lead compound, 1,2,3,4,6-penta-*O*-galloyl-β-*D*-glucose (PGG), was protective in vitro and in cell-based assays, and similar tannins are known to be inhibitors of cholera toxin (see Figure 3).[105] Other sugar-based molecules such as guanidinylated saccharides[169,170] were also found to have submicromolar potencies against LF in vitro and in cell-based assays (Figure 7).

Building off of literature that identified hydroxamates as inhibitors of LF and other metalloproteases, Shoop and colleagues used a combination of screening and medicinal chemistry to identify the inhibitor LFI ((2*R*)-2-[(4-fluoro-3-methylphenyl)sulfonylamino]-*N*-hydroxy-2-(tetrahydro-2*H*-pyran-4-yl)acetamide) as a potent inhibitor of LF (Figure 7).[171,172] LFI competitively inhibited LF with an IC_{50} of 60 nM and a K_i of 24 nM, protected macrophages from LF-mediated toxicity with an IC_{50} of 160 nM, and was efficacious in a toxigenic mouse model. In a mouse model with the Sterne strain of *B. anthracis*, mice were continuously infused with 250 $\mu g \times 100\ \mu L^{-1} \times h^{-1}$ LFI via jugular vein catheter starting 24 h before *B. anthracis* challenge. While 100% mortality of saline-treated mice was achieved by day 3 postinfection, 50% of LFI-treated mice survived for the 9 day experiment. While this result showed positive effects of LFI on live *B. anthracis* infection, continuous infusion of LFI was required due to pharmacokinetic data indicating a short half-life of 0.4 h for LFI in mice, a concern for a potential therapeutic lead. In a rabbit model of *B. anthracis* infection, subcutaneous treatment with 100 mg/kg LFI three times a day for 7 days led to a 50% survival (2/4) over the 21 day experiment when LFI treatment was initiated at the same time as *B. anthracis* spore challenge. Importantly, when treatment was initiated 1 day after spore challenge for a total of 6 days of treatment, one of four rabbits survived. Further, in the same rabbit model of *B. anthracis* infection, treatment with a combination therapy of ciprofloxacin (5 mg/kg subcutaneously (sc), two times a day for 2 days) and LFI (100 mg/kg sc, four times a day for 1 day) could be delayed up to 66 h postinfection with 100% survival (4/4). Interestingly, peritoneal cultures of the rabbits indicated that the animals in the ciprofloxacin group were negative for *B. anthracis* infection, highlighting the limitation of antibiotic treatment in anthrax infection, as bacterial clearance was not sufficient to save two of the four animals. While optimization of LFI would need to be carried out to improve its pharmacokinetic profile before initiating human clinical trials, this compound is the most advanced in terms of protective effect in the context of in vivo infection. Further, experiments in the rabbit model of infection clearly show its potential to be used in combination with standard of care antibiotics to improve outcomes.

The hydroxamate reported by Shoop and colleagues was used as a starting point for medicinal chemistry efforts by Jiao and colleagues.[173] A benzyl amine derivative of a 3,5-dimethyl-4-fluorobenzene core structure (PT-8541) had an in vitro K_i value of 0.05 nM, a >500-fold improvement over the in vitro K_i of LFI (Figure 7). In an in vivo rat intoxication model, doses as low as 5 mg/kg of the benzene resulted in 100% survival.[174] Additional studies explored further modifications[175] and showed efficacy of these small-molecule inhibitors as a cotreatment with subtherapeutic doses of antibiotics or antibodies in mouse models of infection,[176] again highlighting the potential of antivirulence agents to be used in combination therapies.

Additional compounds utilizing hydroxamates have been reported in the literature, including D-proline peptidomimetic hydroxamic acids (Figure 7).[177] Structural studies with D-alanine-derived hydroxamates identified a deep binding pocket that was targeted with P1′ aromatic substituents.[178,179] Follow-up studies by the same group investigated further optimization of hydroxamate inhibitors to bind to a solvent-exposed S2′ binding pocket.[180]

Small-molecule inhibitors that chelate the zinc ion using moieties other than hydroxamates have also been reported. Forino et al. used a fragment-based approach focusing on drug-like scaffolds to identify small-molecule inhibitors bearing a core phenylfuran-2-ylmethylenerhodanineacetic acid scaffold (B1-11B1) with in vitro K_i values between 32 and 300 nM (Figure 7).[181] In an in vivo model using *B. anthracis* challenge, postexposure treatment with B1-11B1 and the antibiotic ciprofloxacin 24 h after infection resulted in 40% survival, compared to 20% survival with ciprofloxacin treatment alone. Another zinc-chelating moiety, catechol, was identified by

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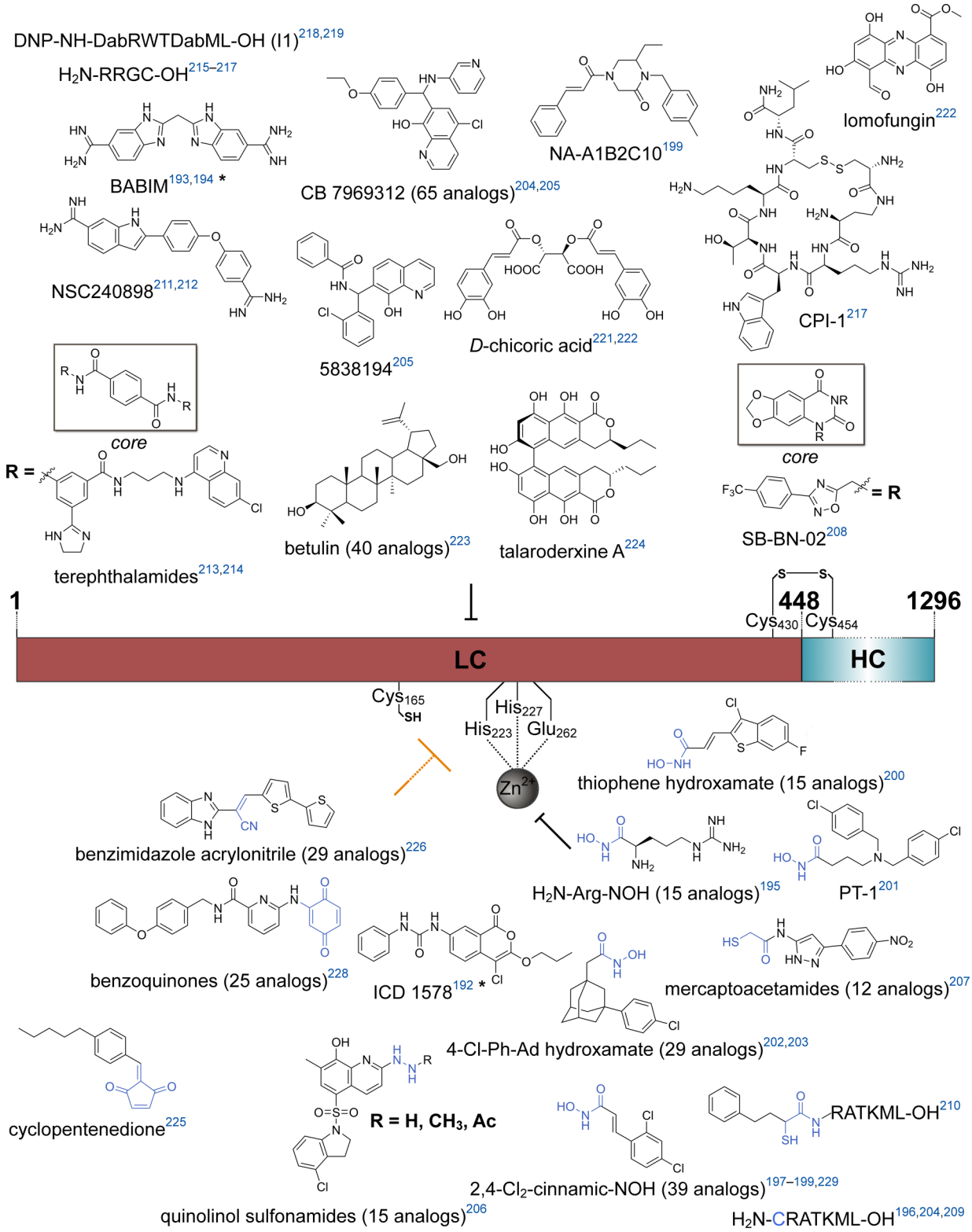

Figure 8. Inhibitors of the light chain metalloprotease (LC) of the *C. botulinum* toxin (BoNT). The LC (red) and the heavy chain (HC; blue) are connected by a disulfide bridge that is reduced after toxin internalization. Numerous hydroxamates bind to the catalytic LC metal binding site created by His_{223}, His_{227}, and Glu_{262} (lower right). Metal chelating sites are shown in blue. Several classes of molecules irreversibly modify the nucleophilic Cys_{165} adjacent to the active site (lower left), and one compound (cyclopentadione) binds the toxin irreversibly by an unknown mechanism. Reactive electrophilic functional groups are shown in blue. Compounds targeting BoNT B only are indicated (∗).

Gaddis et al. using a medium-throughput cell-based screen to identify LF inhibitors.[182] The small molecule *N*-oleoyl-dopamine (OLDA) was found to inhibit LF activity in situ and in vitro in the single digit micromolar range (Figure 7).

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To find novel scaffolds that inhibited LF activity, Johnson and colleagues screened 16 000 drug-like molecules using a fluorescent peptide cleavage HTS, identifying six compounds with IC_{50} values under 20 μM.[183] Significant SAR analysis of one lead hit, a small molecule containing a thiophene residue to coordinate the zinc ion, indicated that benzothiazole thiophenes (BTTs) were more potent than benzothiazole-4-methylthiophenes (BTMTs) and introduction of biphenylsulfonyl groups also improved potency. The most potent inhibitor with favorable pharmacokinetic properties, *N*-(3-(benzo[*d*]-thiazol-2-yl)isoazol-5-yl)-4′-methoxy-biphenyl-4-sulfonamide (BTT-70), protected cells against LF-mediated toxicity with an EC_{50} of 506 nM, and displayed a modest protective effect in a mouse model of inhalational *B. anthracis* when treated daily with 25 mg/kg beginning 1 day after infection (Figure 7).

Small-molecule inhibitors with many different mechanisms of action have been developed for anthrax. An attractive option is to develop PA inhibitors, since PA is a required component of both LT and ET, and these inhibitors do not need to be cell-permeant. Multivalent compounds have been most successful in inhibiting PA. Another promising approach is LF inhibition. Significant efforts have yielded compounds with high potency and successful in vivo efficacy in animal models. Further, some inhibitors targeting LF have also been used with antibiotics, highlighting the benefits of combination therapy. Many of these compounds hold significant promise to advance to the clinic. However, due to the rare nature of anthrax infection and high mortality rates, the path to clinical approval is unclear.

3.9. *Clostridium botulinum*

The spore-forming, obligate anaerobe *C. botulinum* produces the most deadly toxin known to man, with a lethal dose of approximately 1 ng/kg.[184,185] Infections are infrequent with less than 200 cases reported per year in the United States, and natural infections typically occur when *C. botulinum* is taken up into a wound or into the gastrointestinal tract.[184] Botulinum neurotoxin (BoNT) is produced by the bacterium and released into the circulation, where it targets presynaptic cholinergic ganglia and motor neurons, causing a symmetric, descending, flaccid paralysis.[184] Treatment is largely supportive and depends on the severity of symptoms, with death occurring in 5−10% of patients, usually via suffocation due to paralysis of upper airway muscles and the diaphragm.[184,186] While infection by this soil-dwelling bacterium is uncommon naturally, it is a significant threat as a weapon of bioterrorism and is listed as a Category A Bioterrorism Agent by the CDC.[187] Aerosolized forms of botulinum toxin have been developed by many nations and organizations, such as Japan, the United States, the former Soviet Union, Iraq, Iran, North Korea, and Syria.[184]

Eight serotypes of BoNT exist (A−H), of which BoNT A is the most relevant serotype for human infection.[188,189] While the serotypes differ significantly in their primary structure, all BoNT serotypes are synthesized as a 150 kDa precursor protein that is proteolytically cleaved into a heavy chain (HC) and light chain (LC), which remain attached by a disulfide bond.[189] The C-terminus of the HC (H_C) consists of a receptor-binding domain, while the N-terminus of the HC (H_N) is a translocation domain.[188,189] Upon H_C-mediated binding to receptors on the neuronal membrane, the toxin is endocytosed, and endosomal acidification induces H_N to form a pore to translocate the LC to the cytosol where it exerts its cytotoxic effects.[188] All BoNT LCs are zinc-dependent metalloproteinases with the conserved catalytic zinc-binding domain HExxH that cleaves soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Of these SNARE proteins, synaptosome associated protein 25 (SNAP-25) is cleaved by BoNTs A and E; synaptobrevin is cleaved by BoNTs B, D, F, and G; and syntaxin and SNAP-25 are both cleaved by BoNT C.[188,189] SNARE proteins act as molecular zippers that facilitate membrane fusion and extrusion of the neurotransmitter acetylcholine, and their cleavage by BoNTs prevents the transmission of action potentials to muscle, resulting in flaccid paralysis. BoNT specificity is due to the presence of exosites that bind residues of the substrate far from the scissile bond and create a long protein−protein interaction between the LC and SNARE protein, requiring 66 amino acids for optimal catalytic activity in the case of BoNT A.[188]

Therapies to treat botulism are limited. Antibiotics are not indicated as a therapy except in cases of wound botulism.[184] One vaccine, the pentavalent (ABCDE) botulinum toxoid (PBT) vaccine, was used for many years for military and laboratory personnel with high risk of exposure. PBT was never clinically approved and remained under the classification of an investigational new drug (IND) until discontinuation of the IND on May 31, 2012 by the CDC due to decreased efficacy and serious adverse effects.[190] Two antitoxins exist to provide passive immunity to infected patients. The equine-derived heptavalent botulism antitoxin is only indicated for adults due to potential adverse effects in infants.[191] For infant botulism, a human-derived botulinum immune globulin, called BIG-IV or BabyBIG, is available.[191] While these antibodies effectively bind toxin in circulation, they cannot act once BoNTs are intracellular, so their efficacy decreases as infection progresses. Due to the long half-life of BoNTs intracellularly, strategies that target the toxin after host cell internalization are needed.

3.9.1. *C. botulinum*: Inhibitors of the Metalloprotease Domain of BoNT B. The majority of the antivirulence agents developed for BoNTs target and inactivate the LC metalloprotease activity. While most of these compounds target BoNT A LC, some have also been developed for the BoNT B LC. Early studies to develop inhibitors of BoNT B metalloprotease activity made use of small molecules previously designed to target other enzymes with similar mechanisms, such as matrix metalloproteases (MMPs). The chloroisocoumarin ICD 1578 (7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin), originally identified as an MMP inhibitor, was found to inhibit BoNT B LC activity with an IC_{50} value of 27.6 μM (Figure 8).[192] In another study, bis(5-amidino-2-benzimidazolyl)methane (BABIM), originally identified as a small-molecule inhibitor of trypsin,[193] was cocrystallized with BoNT B (Figure 8).[194] This zinc chelator was reported to have an IC_{50} of 5−10 μM against BoNT B LC, and two molecules of BABIM complexed with the holotoxin in the crystal structure. The two BABIM molecules partially occluded the synaptobrevin substrate binding site, and kinetic soaking experiments indicated that the zinc ion was removed in a time-dependent manner, leading to a disordered active site conformation. Further in vitro and in vivo studies are required to determine which of the potential mechanisms of action are responsible for toxin inhibition and in vivo efficacy, but this study did show that small-molecule inhibitors of LC activity could also bind the holotoxin, therefore potentially acting both extracellularly and intracellularly.

3.9.2. *C. botulinum*: Inhibitors of BoNT A. *3.9.2.1. C. botulinum: Zinc Chelating Inhibitors.* Most small-molecule antitoxin agents have been developed to inhibit BoNT A,

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considered to be the most relevant botulinum neurotoxin for human infection, as well as the serotype used in many commercial Botox preparations.[189] By developing a FRET-based high-throughput screening assay with a 13-mer truncated substrate, SNAPtide,[195,196] Boldt and colleagues screened a library of hydroxamate-based compounds (Figure 8).[195,197] From the initial hit, *p*-chlorocinnamic hydroxamate, with an in vitro IC_{50} value of 15 μM, further medicinal chemistry was carried out to generate 12 analogues. This SAR analysis revealed the importance of the *p*-chloro substitution, and led to an analogue with an *o,p*-chloro substitution, 2,4-dichlorocinnamic hydroxamate, with an IC_{50} value of 0.41 μM (Figure 8). Further SAR analyses identified three analogues with substitutions at the *ortho* position with similar potency.[198]

In another follow-up study, a HTS of ~66 000 compounds was carried out by the same group, with a secondary cell-based screen used to further stratify hits.[199] Seven compounds with in vitro IC_{50} values ranging from 1.5 to 91.2 μM and 61−25% cellular protection against SNAP-25 cleavage were chosen for testing in an in vivo mouse lethality assay, in which 5−10 times the LD_{50} of BoNT A was administered intraperitoneally and animals were monitored for time to death. The previously identified 2,4-dichlorocinnamic hydroxamate[197] was also included as an eighth compound, though, interestingly, in situ cell-based studies showed that 2,4-dichlorocinnamic hydroxamate was toxic to cells at 5 μM and above, and did not protect against toxin-induced SNAP-25 cleavage below cytotoxic concentrations. Intravenous treatment with 100 or 250 nmol of two compounds, 6-ethyl-1-[(4-methylphenyl)methyl]-4-[(2*E*)-1-oxo-3-phenyl-2-propen-1-yl]-2-piperazinone (NA-A1B2C10) and 2,4-dichlorocinnamic hydroxamate, showed partial efficacy in vivo (Figure 8). NA-A1B2C10 increased time to death by 36% compared to controls (average time of death 484 min for controls versus 659 min for NA-A1B2C10-treated mice), while 16% (5/31) of mice treated with 1 mM 2,4-dichlorocinnamic hydroxamate displayed no symptoms of botulism and survived the challenge, although no increase in time to death was seen in the animals that did not survive. This study showed that in vitro and in situ models of botulism intoxication may not directly translate to in vivo models of botulism.

To address the apparent disconnect between in vitro and in vivo efficacy, Čapek and colleagues optimized 2,4-dichlorocinnamic hydroxamate to improve its pharmacokinetic properties and create a drug-like inhibitor.[200] Two moieties of the parent compound were systematically replaced by substituting the hydrophobic 2,4-dichlorophenyl ring with benzothiophene and the labile cinnamate with methylation or reduction of the double bond to create derivatives with similar nanomolar potency as the parent molecule (Figure 8). Interestingly, while compounds that replaced both moieties only had a modest decrease of in vitro potency, they displayed higher rates of metabolic breakdown and a shorter serum half-life than the parent. In contrast, the benzothiophene derivative retaining the cinnamic double bond ((2*E*)-3-(3-chloro-6-fluoro-benzo[*b*]-thiophen-2-yl)-*N*-hydroxy-(*E*)-acrylamide) displayed a 6-fold improvement over the parent in its in vitro potency with a K_i of 77 nM, as well as increased serum stability and half-life. While this study highlights the ability to make hydroxamate small-molecule inhibitors more drug-like with improved pharmacokinetic properties, their overall hydrophobicity remains a concern, as a high amount of circulating drug will likely be bound to plasma proteins resulting in poor bioavailability. In addition to the hydroxamates discussed above, branched-scaffold hydroxamate inhibitors (PT-1) with in vitro IC_{50} values in the low micromolar range[201] as well as hydroxamate-based compounds coupled to adamantane[202] have also been described (Figure 8).

Overall, studies identifying hydroxamate-based inhibitors showed that stability, cellular uptake, and toxicity were significant challenges, as some compounds with good in vitro potency were not efficacious or were toxic in in situ or in vivo studies. One strategy employed by Seki et al. to circumvent this issue was to create a prodrug with selective O-carbamation of the hydroxamate.[203] Although this strategy was partially successful at decreasing the cytotoxicity in a cell-based assay, the compounds were only weakly potent, with EC_{50} values in the high micromolar range.

In addition to using hydroxamate scaffolds to chelate the zinc ion, diverse chemical scaffolds have been used. Using in silico screening of >250 000 compounds from the National Cancer Institute (NCI) database and an HPLC-based secondary in vitro assay, Roxas-Duncan et al. identified a quinolinol scaffold with in vitro efficacy against BoNT A.[204] Because the originally identified compound 7-((4-nitroanilino)(phenyl)methyl)-8-quinolinol (NSC 1010) displayed neurotoxicity, a follow-up screen of 55 analogues identified five nontoxic analogues with improved potency over the parent compound. The lead compound, 5-chloro-7-[(4-ethoxyphenyl)(3-pyridinylamino)-methyl]-8-quinolinol (CB 7969312), displayed an in vitro IC_{50} of 2.1 μM, completely protected SNAP-25 from cleavage in a cell-based assay at 10 μM, and offered a 4-fold increase in protection against toxin-induced muscle paralysis in an ex vivo mouse phrenic nerve hemdiaphragm assay (271 min average time to 50% loss of twitch tension with CB 7969312 compared to 65.7 min for controls) (Figure 8). Building off the quinolinol scaffold, a focused screen of 188 quinolinol compounds identified 8-hydroxyquinoline derivatives (such as 5838194) with IC_{50} values around 1 μM (Figure 8).[205] This study also identified two clinically approved compounds that are safe in humans and display good pharmacokinetic properties, cliquinol and chloroxine, which showed modest activity against BoNT A LC. Moving into human clinical trials with already approved compounds is one viable strategy to generate efficacy data for antivirulence compounds. Initial pharmacokinetic studies of lead quinolinols suggested that these compounds display good serum half-lives but extremely poor solubility in neutral pH. Recent studies have further diversified the 8-hydroxyquinoline scaffold with sulfonamide substituents as BoNT A LC inhibitors (Figure 8).[206] Other scaffolds, including mercaptoacetamides that use the thiol to coordinate the zinc ion,[207] and quinazolines (such as SB-BN-02) that inhibit the BoNT A LC with an undefined mechanism,[208] have been reported with low micromolar potency in vitro (Figure 8).

3.9.2.2. C. botulinum: Substrate Mimic Inhibitors. An alternate approach to using zinc chelators to block BoNT function is the use of substrate mimics to block metalloprotease activity. The first specific BoNT A small-molecule peptide inhibitors were designed based on the amino acid sequence of the SNAP-25 cleavage site, with substitutions of L- and D-cysteine residues to coordinate the zinc ion.[209] Substitution of the P1 glutamine of the SNAP-25 substrate for L- or D-cysteine yielded the best inhibitors, with smaller peptides being more potent than the initial 17-mer library tested. The most potent inhibitor, CRATKML, demonstrated competitive inhibition with an in vitro K_i of 2 μM (Figure 8). Another group used

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CRATKML in a mouse phrenic nerve hemidiaphragm assay and reported no protection against toxin-induced muscle paralysis compared to control.[204] In a follow-up study with CRATKML, the cysteine residue was replaced with sulfhydryl-containing moieties.[210] Replacement of the N-terminal cysteine with 2-mercapto-3-phenylpropionyl followed by RATKML (mpp-RATKML) yielded a stereoisomer that competitively inhibited BoNT A with a K_i of 330 nM (Figure 8). Further, the C-terminal residues after Met had no effect on inhibitor binding, indicating that the mmp-RATKM residues were the most important for inhibition of metalloprotease activity. By modeling mpp-RATKML onto the solved structure of BoNT A LC, Burnett and colleagues characterized the pharmacophore for BoNT A LC inhibition to screen for non-peptidic small-molecule inhibitors.[211] Searching for molecules that fit this pharmacophore, four compounds were identified with K_i values from 3.0 to 10.0 μM. While NSC341909 (K_i = 3.0 μM) and NSC308574 (K_i = 6.0 μM) were cytotoxic, NSC240898 (K_i = 10.0 μM) was permeable to chick neurons, was not cytotoxic up to 40 μM, and showed a dose-dependent, partial inhibition of SNAP-25 cleavage in situ (Figure 8). In several follow-up studies by the same group, the NSC240898 scaffold was optimized using virtual library screening,[212] medicinal chemistry,[213] and pharmacophore-based design[214] to identify terephthalamide-based inhibitors with nanomolar K_i values in vitro (Figure 8). The significant optimization of these non-peptidic small-molecule inhibitors resulted in compounds with similar potency as the original peptidic inhibitor that are promising scaffolds for therapeutic leads.

Using cocrystallography and peptide-based substrate mimics, Kumaran et al. also evaluated the importance of specific residues in substrate-based peptidic inhibitors.[215] By characterizing the binding pocket of BoNT A, the optimal tetrameric peptide was designed to contain positively charged residues at the N-terminus to interact with the negatively charged zinc-binding region, and hydrophobic residues at the C-terminus to interact with hydrophobic loops of the enzyme. Four tetramers, RRGC, RRGL, RRGI, and RRGM, had in vitro IC_{50} values of 157, 660, 786, and 845 nM, respectively, although the most potent RRGC peptide was significantly less potent in the presence of $ZnCl_2$ and DTT (Figure 8). These tetramers mimicked the native cleavage site of SNAP-25, with P1 and P1′ residues of Gln^{197}-Arg^{198}, where Arg replaced Gln for additional positive charge at the N-terminus of the tetrapeptide. Interestingly, the authors found that increasing the length of the inhibitor to a hexapeptide decreased potency dramatically, with substrate mimics QRATKM and RRATKM having in vitro IC_{50} values of 133 and 95 μM, respectively.[216] Based on these peptide sequences, the same group synthesized a cyclic peptide inhibitor (CPI) with the sequence C(DAB)RWTKCL cyclized with a disulfide bond between the P2 and P5′ residues (Figure 8).[217] Cocrystallization revealed that the 2-amino group of DAB replaced the nucleophilic water coordinating with the catalytic zinc, and the cyclized peptide occupied more of the BoNT A active site than linear counterparts, inhibiting metalloprotease activity in vitro with a K_i of 12 nM. However, CPI showed limited efficacy in cell-based and in situ assays, likely due to low permeability and stability in vivo, a significant drawback of all peptide-based inhibitors.

In another structure-based study, residues in the core substrate sequence were substituted with non-peptidic moieties to create a peptidomimetic inhibitor, I1.[218] The most potent 7-mer inhibitor, DNP-DabRWTDabML (I1), had an in vitro K_i of 41 nM and displayed no inhibitory activity against BoNT B, D, E, or F or thermolysin (Figure 8). While no in vivo data was reported for this compound, substitution of amino acid residues with synthetic moieties created a more drug-like compound that could be further used as a scaffold to develop therapeutics to test in in vivo systems. Follow-up studies to further derivatize I1 did not yield compounds more potent than the parent I1 inhibitor.[219]

Using epitope targeting and target-guided synthesis, Farrow and colleagues designed an inhibitor of BoNT A that recognized the active site as well as an adjacent solvent-exposed region of the protease.[220] The substrate mimic Dab(DNP)-R-Lys(N_3)-T-Dab-Pra-L-R (Inh-1) targeting the active site was found to have an in vitro K_i of 70 nM. To identify a potent binder of the adjacent solvent-exposed epitope on BoNT A, a macrocyclic peptide library of 1 100 000 5-mer peptides was screened. The resulting hit, R^2-Pra-NYRWL-Lys(N_3) (L2), was chosen with a K_D of 78 nM and no inhibition of the LC. A combinatorial linker library selected the consensus sequence Gly-Aib-Leu to connect Inh-1 and L2, resulting in the inhibitor Inh-2 with an in vitro K_i of 165 pM. Inh-2 displayed in situ protection against SNAP-25 cleavage in neuronal cells at concentrations as low as 100 nM, and partially rescued neurons from BoNT intoxication up to 3 h post toxin exposure at doses of 1 μM. However, Inh-2 is cell-impermeant and relies on the holotoxin or a spontaneously translocating peptide (STP) to shuttle it into the cell, a drawback for its development into a therapeutic and for postexposure treatment. Additionally, in situ assays measured efficacy after 24 h, and therefore may overestimate the protective effects of the compound, as BoNT is highly persistent in neurons. Nevertheless, Inh-2 is a highly potent compound that warrants further development and optimization.

3.9.2.3. C. botulinum: Natural Product Inhibitors. In a unique strategy to target BoNT intoxication upstream of intracellular LC activity, Fischer et al. explored the natural product toosendanin and its analogues as inhibitors of botulism (Figure 1).[77] This natural product from the *Melia azedarach* var. *toosendan* plant was used as a traditional Chinese remedy for botulism, but its mechanism of inhibition was unknown. In a cell-based spinal cord assay, toosendanin completely blocked SNAP-25 cleavage at 200 nM, with an ED_{50} of 55 nM. However, up to millimolar concentrations, toosendanin did not inhibit BoNT A LC activity. Toosendanin was efficacious in blocking BoNT E toxicity in a cell-based assay, leading the authors to hypothesize that it acted on BoNT internalization. Using excised membrane patches, toosendanin blocked LC translocation by H_N in a dose-dependent manner with an ED_{50} of 4 nM. Further, addition of toosendanin after LC translocation resulted in a change of H_N kinetics, increasing the time in which the channel is in its open state in a dose-dependent manner. THF-toosendanin was found to be approximately 10-fold more potent in blocking LC translocation. In a mouse lethality assay, 250 nmol of toosendanin and THF-toosendanin administered IV increased the time to death 4-fold (7.1 h in controls versus >28 h in inhibitor-treated animals), in accordance with previous reports of toosendanin protection in this assay. This compound was later found to inhibit endosomal acidification when tested in anthrax and *C. difficile* infection, in addition to a potential secondary synergistic mechanism of inhibition. The bimodal activity of toosendanin and its analogues in botulism, its activity in vivo, as well as its efficacy against anthrax and *C. difficile* infections, make it an

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attractive therapeutic lead that could potentially act on multiple bacterial toxins.

Another natural product was studied by Silhar et al. for its effect against BoNT A.[221] Phenolic derivatives of the echinacea plant were found to partially inhibit BoNT A in vitro with a noncompetitive partial inhibition mechanism, indicating binding at an exosite. The compound D-chicoric acid inhibited BoNT A with a competitive inhibition constant of 0.7 μM (Figure 8). However, this compound saturated at 60% inhibition, and the authors did not identify an exosite binding location. While the lack of complete inhibition likely precludes further development as a drug lead, the authors note that exosite inhibitors could be used synergistically with active site inhibitors to improve neuronal protection. In a similar follow-up study, the metabolite lomofungin from the gram-positive bacterium *Streptomyces lomodensis* also displayed noncompetitive inhibition of BoNT A LC (Figure 8).[222] While the authors could not identify the specific binding site, they hypothesized that it binds to an exosite separate from that of the D-chicoric acid binding site due to the fact that inhibition was not mutually exclusive between the two compounds. In a neuronal assay, lomofungin displayed weak potency with an EC_{50} of 131 μM. While these natural products are intriguing in their potential mechanism of inhibition, further studies need to be conducted to identify their binding sites. Characterization of these binding sites could also be used to further elucidate the specificity elements of BoNT A, making these compounds potential tools to uncover novel biology of BoNT A pathogenesis. In another study, 28-hemisuccinylbetulin, a derivative compound of a natural extract of *Betula* spp. outer birch bark, had a high nanomolar K_i against BoNT A metalloprotease activity but failed to protect cells in an in situ assay (Figure 8).[223] The authors predicted the lack of cellular efficacy to be due to poor solubility. Natural products produced by fungal species also have activity against BoNT A LC. Using an in silico screen of 350 000 compounds, Cardellina et al. identified highly aromatic fungal metabolites based on bis-naphtho-γ-pyrones such as talaroderxine A with low micromolar IC_{50} values in vitro (Figure 8).[224] These compounds also had efficacy in situ in the mouse phrenic nerve hemidiaphragm assay. Together, these natural products represent novel scaffolds for development of potent compounds against BoNT A LC.

3.9.2.4. C. botulinum: Irreversible Inhibitors of the Metalloprotease Domain. Another approach to target BoNT A is to develop irreversible small-molecule inhibitors. This strategy is attractive due to the long half-life of BoNTs in neurons and the lack of toxin regeneration, allowing a covalent inhibitor to bind to and inactivate the total pool of toxin. The first irreversible covalent inhibitors of BoNT A were reported by Capkova and colleagues,[225] using scaffolds based on their previous work with hydroxamate inhibitors.[197,198] Substituting the hydroxamate with a cyclopentenedione warhead yielded two irreversible 4-cyclopentene-1,3-dione inhibitors with k_{inact}/K_I values of 520−580 M^{-1} s^{-1} (Figure 8). However, the authors were unable to determine the residue of covalent modification, making the mechanism of these compounds unknown. Additionally, the compounds only provided protection in mouse primary neurons at concentrations of 600−900 μM and were not stable in serum, limiting their potential as drug leads. In another study, Li et al. performed a FRET HTS of 70 000 compounds and identified a benzimidazole acrylonitrile with an IC_{50} of 7.2 μM (Figure 8).[226] Subsequent SAR analysis led to identification of an analogue with a bisthiophene substituent, displaying a moderately weaker IC_{50} of 26 μM but with improved specificity over the original hit. Further, the bisthiophene analogue was more potent in a neuronal assay, inhibiting 58% of SNAP-25 cleavage at 30 μM concentration, in comparison to 10% inhibition at the same concentration of the parent compound. These compounds also displayed time-dependent inhibition, leading the authors to hypothesize that these compounds covalently modified the cysteine 165 residue adjacent to the active site. We speculate that the zinc-binding bisthiophene positions the potential warhead close to Cys165, facilitating nucleophilic attack. While modeling and docking studies supported this hypothesis, cocrystallization or mass spectrometry would confirm the site of modification. Subsequent mutagenesis studies found that Cys165 mutation decreased catalytic efficiency 50-fold, providing support that this residue is important for BoNT A LC catalysis, and validating the rationale for targeting this residue by small-molecule inhibitors.[227] Targeting Cys165 with benzoquinone derivatives was employed by Bremer and colleagues.[228] They identified 2,5-dichlorobenzoquinone with a k_{inact}/K_I of 84 M^{-1} s^{-1}; however, when a rational, fragment-based approach to link the benzoquinone to a zinc chelating moiety was attempted, the inhibitors displayed competitive and reversible inhibition. 1,4-Benzoquinones linked to picolinamide (Figure 8) chelating moieties resulted in IC_{50} values of 2.3−2.5 μM for the top two hits, but highlighted the difficulty in designing covalent, irreversible inhibitors of BoNT A utilizing multiple binding modes.

The body of literature on antivirulence compounds for BoNTs shows that there are multiple potential strategies to target this metalloprotease: zinc chelation, substrate mimicry, and targeting catalytic or nearby nucleophilic residues with covalent inhibitors. Additionally, extensive structural characterization, both of the apoenzyme and cocrystals with bound inhibitors, can aid in the development of rationally designed inhibitors. These structural studies have also revealed a significant amount of flexibility in the BoNT LC active site,[219,229] indicating that potentially all of these strategies, or a combination of strategies, could be effective methods. However, despite the significant efforts to develop small-molecule inhibitors for BoNTs, none have advanced to human clinical trials. Targeting and gaining access to neurons where BoNTs exert their cytotoxic effects, the long half-life of the toxins in neurons, and the presence of exosites important for specificity all remain significant challenges to the design of compounds with sufficient potency, selectivity, and pharmacokinetic properties for use in humans. Further, few compounds have been tested in vivo, and one study challenged the ability of in vitro studies to correlate with in vivo efficacy,[199] highlighting the importance of in vivo studies to fully assess the potential of these compounds to advance into the clinic.

3.10. *Clostridium tetani*

Tetanus is typically caused by bacterial infection with *C. tetani* through a break in the skin.[230] Classical symptoms begin with lockjaw and spread to the body, where patients experience muscle spasms and spastic paralysis.[230] Tetanus vaccines and postexposure prophylaxis with antibodies have reduced the number of infections in developed countries, but infant tetanus remains a large concern in developing countries. Tetanus caused an estimated 213 000 deaths worldwide in 2002, a large majority of which were cases of neonatal tetanus.[230]

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Tetanus toxin (TeNT, also known as tetanospasmin) is a single 150 kDa peptide that is cleaved into a heavy chain (100 kDa) and light chain (50 kDa) that remain linked by a disulfide bond. The heavy chain mediates receptor binding, internalization, and transport down the neuronal axon, after which the light chain is released into the cytosol at the axon terminal. The light chain functions as a zinc metalloprotease that cleaves synaptobrevin, a protein that functions in a complex of proteins that act as a molecular zipper fusing neurotransmitter-filled vesicles with the plasma membrane. Because TeNT is more specific for inhibitory than excitatory neurons, intoxication with TeNT leads to the loss of inhibitory neuronal signals and patients display spastic paralysis.

3.10.1. *C. tetani*: Inhibitors of Toxin Internalization. Using bafilomycin A1, an inhibitor of the vacuolar-type H^+-ATPase that acidifies endosomes, and brefeldin A, an inhibitor of Golgi function, as pharmacological tools, Williamson and Neale showed that TeNT required acidified endosomes for translocation into the cytosol and was not trafficked through retrograde transport (Figure 1).[231]

3.10.2. *C. tetani*: Inhibitors of the Metalloprotease Domain. In the first study to identify modestly potent inhibitors of the light chain metalloproteinase activity, Martin et al. used the peptide sequence of synaptobrevin to design a series of substrate mimics.[232] Iterative SAR analysis of the natural cleavage site led to the identification of the most potent compound, β-amino-(4-sulfamoylphenyl)glycine-thiol with an in vitro K_i of 35 μM. The primary amine, a zinc-chelating sulfonyl group, and the sulfonamide were determined to be key moieties for the potency of this compound. Follow-up studies by this same group used combinatorial libraries of pseudotripeptides (Figure 5B) to improve the potency of the parent inhibitor to a K_i of 3 μM by adding the most favorable P1′ and P2′ moieties, tyrosyl-histidyl, capped with a C-terminal benzylamide (Figure 5B).[233] Further studies to determine cell permeability and stability of these compounds are required before they can be considered as drug candidates, in addition to optimization to improve potency. One reason that the development of small-molecule inhibitors of TeNT may be difficult is that, similar to BoNTs, efficient cleavage of the natural substrate requires 50 residues, likely indicating the presence of exosites that determine specificity. Therefore, while the light chain of TeNT shares over 70% sequence homology with the light chain of BoNT B and 41.3% overall sequence identity for the full length toxin, exosites required for specificity may preclude the use of BoNT inhibitors as scaffolds for the development of TeNT inhibitors.

3.11. *Shigella dysenteriae* Type 1 and Shiga Toxin Producing *E. coli* (STEC)

Drug-resistant *Shigella* spp. are also listed as a serious health threat by the CDC (see Table 1).[6] Responsible for approximately 1/2 million infections per year in the United States alone, symptoms include diarrhea (sometimes bloody), abdominal pain, tenesmus, and fever.[234] Transmission occurs through the ingestion of contaminated food or water, as well as person-to-person contact, with the ingestion of as few as 10 organisms sufficient to cause disease.[235,236] While rare in the United States, one species of *Shigella*, *S. dysenteriae* type 1, contains a chromosomally encoded Shiga toxin (Stx) and is the causative agent of dysentery.[235] Endemic and epidemic *S. dysenteriae* type 1 is a major concern in developing countries that lack clean water, with children ages 1−4 having the highest incidence of infection.[235] Treatment for *S. dysenteriae* type 1 includes supportive care, such as oral rehydration, and antibiotics to decrease the length of infection and transmission to others.[235] However, significant drug resistance to tetracyclines, ampicillin, and trimethoprim-sulfamethoxazole in addition to emerging resistance to fluoroquinolones poses a significant worldwide health threat.[6]

Similar to *S. dysenteriae*, Shiga toxin producing *E. coli* (STEC) is transmitted in contaminated water and food and also produces a highly similar toxin to Stx, Shiga-like toxin 1 (Stx1).[117] STEC can also produce a related toxin, Stx2, which shares 56% sequence similarity to Stx/Stx1. In vitro assays report differences in cytotoxicity, with Stx2 having greater cytotoxic effects on human glomerular endothelial cells.[237] Further, Stx is chromosomally encoded in *S. dysenteriae*, while Stx1 and Stx2 are encoded on prophages that can be transmitted among bacteria, making the distinction between STEC strains that express either or both Shiga-like toxins difficult. The CDC estimates that STEC causes between 175 000 and 265 000 infections in the United States per year.[238,239] Symptoms of STEC infection are similar to that of *S. dysenteriae* type 1, with bloody diarrhea, abdominal pain, and vomiting.[117]

Serious complications and long-term sequelae mediated by Shiga and Shiga-like toxins, including hemorrhagic colitis and hemolytic uremic syndrome (HUS), occur in up to 15% of infections and are concerning features of both *S. dysenteriae* type 1 and STEC infections.[240,241] Shiga and Shiga-like toxins mediate HUS pathology (hemolytic anemia, uremia, and thrombocytopenia) via receptor-mediated binding to glycosphingolipid globotriasylceramide (Gb_3, Gal(α1−4)Gal(β1−4)-Glc(β1−*O*-ceramide)) on the surface of endothelial cells, leading to destruction of the microvasculature.[240,242] HUS is typically most severe in children, where toxin-mediated pathology leads to acute renal failure and can also target small blood vessels in the brain, lungs, gastrointestinal tract, and pancreas.[240,242] Complicating the treatment of STEC infection, and in contrast to *S. dysenteriae* type 1 infection, antibiotic use is controversial; best evidence supports no benefit with antibiotic treatment, and many studies suggest a higher risk for the development of HUS with antibiotic treatment.[241] Antibiotics are contraindicated due to the fact that some may induce expression of Shiga-like toxins and the lysis of many bacteria may cause release of large amounts of toxin, further inducing damage to host cells.[241−243] Therefore, there is an urgent need for toxin-neutralizing therapies that mitigate the damage caused by Shiga-like toxins and reduce long-term complications of HUS, such as hypertension and renal disease.[244]

Shiga toxins are AB_5 toxins that bind to Gb_3 on cell surfaces. Receptor-mediated endocytosis was shown to be important for Stx internalization using primary amines such as ammonium chloride to inhibit toxin uptake.[245,246] Upon binding, the toxin is endocytosed, where the catalytically active A1 domain is cleaved from, but remains disulfide bound to, the A2 domain that binds to the pentameric B subunits. Using the natural product brefeldin A, a lactone antibiotic synthesized by some fungal species, Donta and colleagues showed that, after receptor binding, the toxin goes through retrograde transport through the Golgi apparatus (Figure 1).[247] A known Golgi apparatus inhibitor, brefeldin A protected protein synthesis and inhibited Stx1- and Stx2-induced morphologic changes to HeLa cells in situ. After processing in the Golgi apparatus, toxin is routed to the endoplasmic reticulum where the reducing environment of

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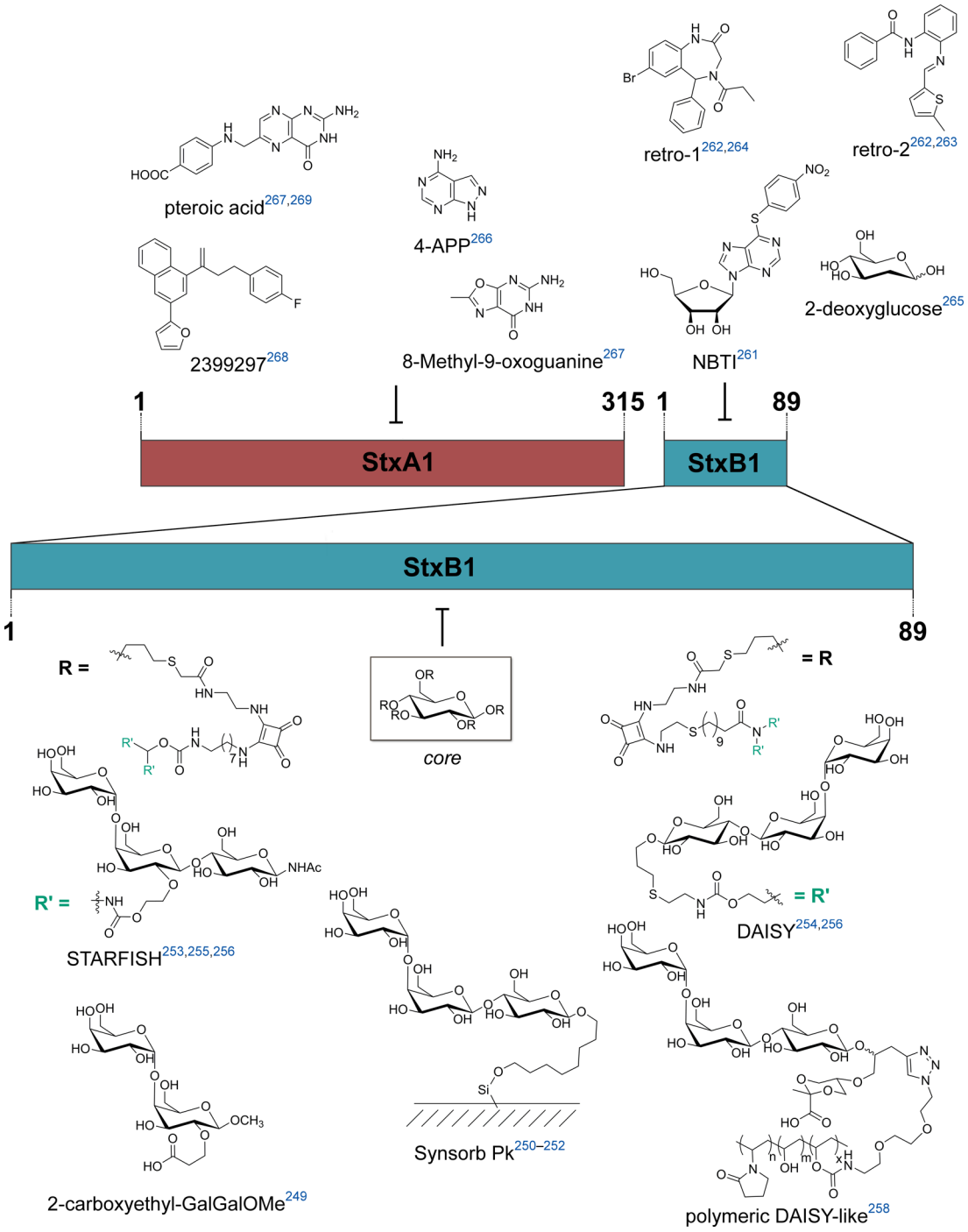

Figure 9. Inhibitors of Shiga toxin Stx from *S. dysenteriae* and the similar Shiga-like toxin Stx1 produced by Shigatoxigenic *E. coli* (STEC). Several compounds target the toxic subunit StxA1 (red) by mimicking NAD^+. Receptor mimics of the native Gb_3 receptor Gal−Gal−Glc trisaccharide containing a core glycan target the binding B1 domain. In some cases, the trisaccharides are linked to a core Glc residue or polymers, resulting in compounds with high valency. NBTI blocks toxin internalization through an alternate mechanism that is not well understood.

the ER allows the A1 fragment to separate and translocate into the cytosol. The A1 fragment acts as an *N*-glycosidase that removes an adenine from the 28S rRNA of the 60S subunit, causing cell death by inhibiting protein synthesis.[248]

While some small-molecule inhibitors have been reported to be efficacious against both Stx/Stx1 and Stx2, the difference in secondary structure likely explains the difference in potency and efficacy for some antivirulence factors, highlighting the high level of specificity of these inhibitors for their targets. Therefore, this section has been divided into two parts: section 3.11.1 focuses on therapies directed primarily toward Stx and Stx1, while section 3.11.2 focuses on small-molecule inhibitors primarily targeted toward the more toxic STEC derived Stx2.

3.11.1. *S. dysenteriae* and STEC: Inhibitors of Stx and Stx1. *3.11.1.1. S. dysenteriae and STEC: Host Cell Receptor Mimics.* The majority of the antivirulence agents designed against Shiga and Shiga-like toxins are carbohydrate-based and modeled after the Gb_3 sugar binding moiety on the host cell. Initial work published by Kitov et al. showed that the free trisaccharides have low millimolar binding affinity for Stx1

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(Figure 9).[249] To improve the potency of these compounds and because each B subunit of the toxin contains three separate binding sites for Gb_3, many of the subsequent carbohydrate-based approaches have coupled lead molecules to polymers to enhance binding to the B pentamer. One of the first polymer-based antivirulence agents designed for the treatment of STEC infection and subsequent HUS is Synsorb Pk (Figure 9).[250−252] Armstrong and colleagues found that the Pk trisaccharide, Gal(α1−4)Gal(β1−4)Glc bearing the same saccharide sequence as the Gb_3 receptor, coupled to the silicon dioxide scaffold Chromosorb P through an eight carbon linker, was preferred by Stx1 over disaccharide sequences in vitro.[250] Synsorb Pk protected Vero cells from *E. coli* extracts containing Stx1, and radiolabeling experiments demonstrated binding of Stx1 to Synsorb Pk. However, Synsorb Pk was less potent at neutralizing Stx2-mediated cytotoxicity. With these positive results, a phase I clinical trial in humans was conducted, in which healthy adult volunteers tolerated oral dosing of 10 g of Synsorb Pk three times daily for 7 days.[251] Further, 25−50% of the dose was recovered from stool and effectively neutralized Stx1 from *E. coli* O26:H11 extracts, with identical efficacy as undigested Synsorb Pk. This indicated that the Synsorb Pk was not being degraded in the gastrointestinal (GI) tract and retains its ability to bind to and neutralize toxin throughout the GI system, especially in the distal intestine where the majority of Shiga toxins are thought to localize. With a clean human safety profile, a randomized, double-blind, placebo-controlled phase III clinical trial in children with HUS was initiated that measured death and serious extrarenal events as the primary outcome measurement (clinicaltrials.gov identifier NCT00004465).[252] The study was discontinued after the second interim analysis, which showed that treatment with 500 mg/kg daily for 7 days failed to decrease the severity of HUS in comparison to placebo. Further development of Synsorb Pk for the treatment of STEC or *S. dysenteraie* was abandoned. There were several limitations that may have precluded success of Synsorb Pk in this study. First, the dose of 500 mg/kg was determined based on efficacy in vitro and in vivo, but may not have been sufficient to bind all toxin. More importantly, only 23% of children had detectable STEC or free Shiga toxin at the time of treatment, and microbiological confirmation of STEC was not included in the inclusion criterion for enrollment in the study. These limitations bring up a number of potential pitfalls: (1) HUS could have been caused by another bacterium, or STEC expressing Stx2, since the toxin type was not specified, and (2) Synsorb Pk's site of action is in the gastrointestinal tract and may have been initiated too late to bind toxin before it bound to its cellular receptor, as indicated by the small percentage of children with detectable bacteria or toxin in the GI tract. These significant limitations to this study design highlight the difficulty in clinical trial design for antivirulence agents and the importance of understanding the pathogenesis of the toxin as well as the mechanism of action of the antivirulence agent to determine the optimal time to initiate treatment.

To improve upon the binding affinity of Synsorb Pk, Kitov and colleagues used X-ray crystallography of the pentameric B subunits to design the multivalent carbohydrate STARFISH (Figure 9).[253] The initial Gb_3 dimer designed to occupy sites 1 and 2 of a B subunit only led to a K_D of 10 μM, but using the structure of the B_5 subunit to design a pentamer of the tethered dimer with a central glucose core and optimal spacing from the center of the pentameric B subunit led to the creation of the STARFISH molecule with an IC_{50} of 0.4 nM against Stx1 and 6 nM against Stx2. Interestingly, cocrystallization of Stx1 B subunit bound to STARFISH revealed that the inhibitor bound to two B pentamers, occupying only Gb_3 binding site 2 of all 10 monomers. STARFISH was unable, however, to protect mice from Stx2 challenge in a lethal intoxication model.[254] To address this inability to neutralize Stx2, Mulvey and colleagues modified the tethering strategy to synthesize DAISY (Figure 9).[254] This multivalent carbohydrate was less potent than STARFISH, with an in vitro IC_{50} of 295 nM against Stx1, and an IC_{50} of 11 μM against Stx2. DAISY and STARFISH differ by their linkages to the main scaffold, with DAISY connected to the linker directly on the reducing end of the trisaccharide while STARFISH is connected by the 2-hydroxyl at the second galactose of the trisaccharide. Additional studies further characterized the binding of DAISY and STARFISH in vitro.[255,256] In a mouse model using an oral *E. coli* O91:H21 strain expressing Stx2, subcutaneous administration of 2 mg of DAISY once a day beginning 24 h after infection led to approximately 50% survival over 10 days. The authors noted that beginning DAISY treatment 48 h after *E. coli* infection abolished protective effects, which likely complicates the use of DAISY as a therapeutic lead for human infection, since patients wait an average time of 2.5−3 days from the onset of diarrhea to an initial doctor visit.[242] Further characterization of the course of infection in mouse and human is required to better design trials that use antivirulence agents that act at an early stage in pathogenesis.

Other Gb_3-based antivirulence agents have been developed, including a compound that targets the Stx1 B subunit and aggregates it to the endogenous protein serum amyloid P component (SAP) through a heterobifunctional multivalent inhibitor.[257] Using a pentavalent core with two ligands to target Stx1 and SAP, this inhibitor-adaptor enhanced toxin inhibition in vitro by aggregating and therefore inactivating Stx1 to endogenous SAP. Alternate scaffolds, such as poly(*N*-vinyl-2-pyrrolidone-*co*-vinyl alcohol), were also used to present Gb_3 ligands on multivalent scaffolds to increase Stx1 binding (Figure 9).[258] Furthermore, screens using poly-NVP-*co*-VA to display Gb_3 ligands at different densities on a microtiter plate were used to design multivalent inhibitors for Stx2.[259] Additionally, different spacer linkages, such as 5-trifluoroacetamido-1-pentanol, have been evaluated as a means to increase inhibitor potency.[260]

3.11.1.2. S. dysenteriae and STEC: Inhibitors of Toxin Internalization. In addition to inhibiting toxin binding, disruption of toxin internalization and trafficking is another strategy used to block Stx-mediated cytotoxicity. Sekino et al. showed that an established nucleoside transport inhibitor, nitrobenzylthioinosine (NBTI), inhibited the intracellular trafficking of Stx1 (Figure 9).[261] Treatment of human renal cortical epithelial cells (HRCECs) with NTBI did not inhibit Stx1 receptor binding or internalization into early endosomes, but inhibited further retrograde trafficking. The authors hypothesized that Stx1 was rapidly excreted after endocytosis in the presence of NBTI. While the exact mechanism of inhibition was not determined, the authors predicted that the ability of NBTI to inhibit nucleoside transport, and its consequent induced decrease in cellular ATP, was responsible for its protective effects. While NBTI showed protective effects in HRCECs at a concentration of 100 μM, higher concentrations were shown to be cytotoxic, likely due to cellular stress induced by decreased ATP levels. Furthermore,

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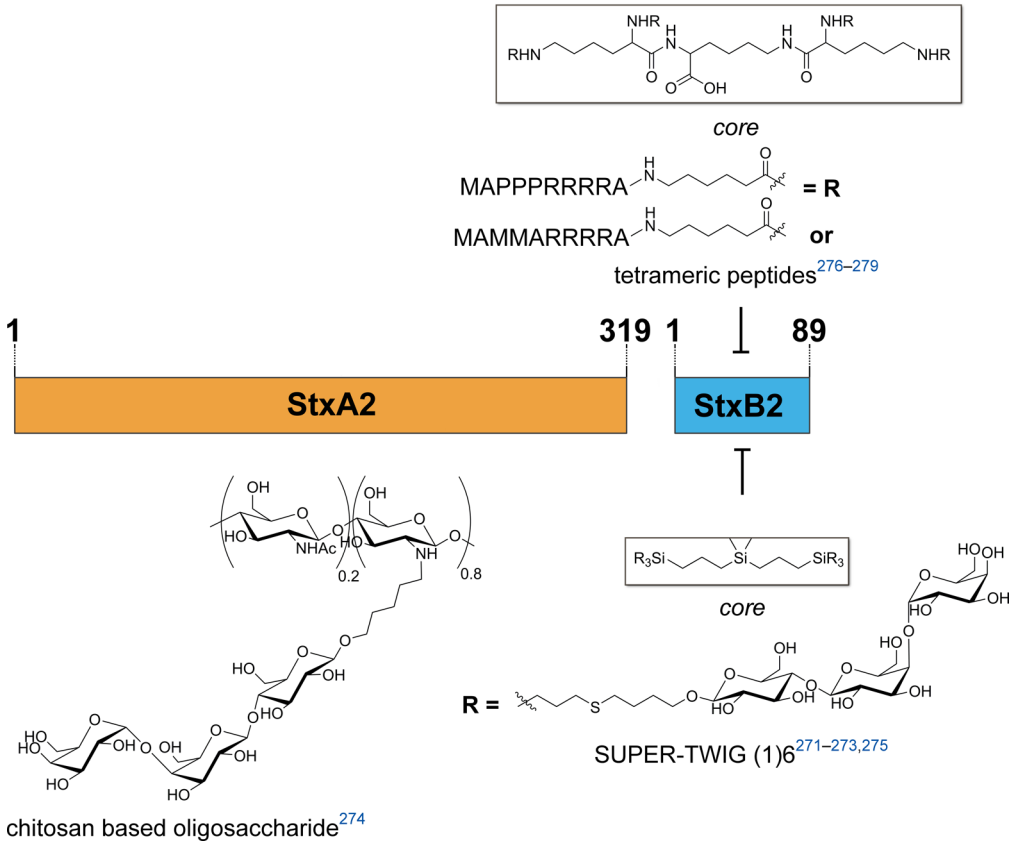

Figure 10. Compounds that target the Shiga-like toxin Stx2 produced by Shigatoxigenic *E. coli* (STEC). Stx2 is an AB_5 toxin that has 56% similarity to Stx/Stx1. The B domain has been targeted by similar approaches as described for Stx/Stx1 (see Figure 9) using multivalent carbohydrates. This core structure can be tethered to carbosilanes or on chitosans as well as other polymeric core structures. Small molecules such as arginine-rich peptides have also been reported as inhibitors for StxB2.

NBTI treatment of HRCECs alone led to a decrease in protein synthesis, making its development as a therapeutic lead unlikely. In another study, Saenz and colleagues performed a cell-based HTS with a luciferase reporter to identify inhibitors of Stx1 internalization.[101] Among the 14 400 compounds that were screened, a benzodiazepine derived molecule (compound **134**) and a scaffold bearing a triazole-imidazole hetereobicyclic core (compound **75**) were identified that reversibly inhibited Stx1 internalization (Figure 1). Using confocal microscopy, as well as testing their effects against other bacterial toxins such as diphtheria toxin (DT) and cholera toxin (CT), compound **75** was found to inhibit toxin transport early in retrograde transport, when toxins are transported to recycling endosomes. In contrast, compound **134** did not inhibit DT, which is not transported in a retrograde fashion through the Golgi and ER, indicating that compound **134** functions later in the retrograde pathway when toxins are transported from recycling endosomes to the Golgi. Another study used a cell-based HTS to screen 16 480 compounds for internalization inhibitors of ricin toxin, a structurally similar *N*-glycosidase.[262] Two structurally diverse compounds, retro-1 and retro-2, bearing a core imine or benzodiazepine scaffold, interfered with retrograde transport to the trans-Golgi network by relocalizing the host protein syntaxin-5 (Figure 9). Retro-1 and retro-2 had an in vitro potency in the low micromolar range for Stx1 and Stx2.[263,264] The nonnatural sugar 2-deoxyglucose was also found to inhibit release of StxA1 from the endoplasmic reticulum (Figure 9).[265] While the small-molecule inhibitors highlighted here are useful tools to further study toxin internalization, all experiments were performed on cells in situ, and it is likely that their targets are not the toxins themselves, but rather host targets important for these trafficking functions.

3.11.1.3. S. dysenteriae and STEC: Inhibitors of N-Glycosidase Activity. Another therapeutic strategy that would act later in the course of infection, after receptor binding and toxin internalization, is targeting the *N*-glycosidase activity of the A subunit directly. Brigotti and colleagues surveyed several purine derivatives to find inhibitors of StxA1 based on substrate mimicry.[266] Using in vitro assays of StxA1 RNA-*N*-glycosidase activity to test purine derivatives as well as a derivative of the purine core structure, 4-aminopyrazolol[3,4-*d*]pyrimidine (4-APP) was identified as an uncompetitive inhibitor of Stx1 that was more potent than adenine (Figure 9). While this compound was not tested in in vivo models, the authors note that while adenine is nephrotoxic in mice, 4-APP is not. Additionally, this reorganization of the atoms in the five-membered ring is a strategy that has also been used to create successful therapeutics, such as allopurinol, an FDA-approved drug that targets the enzyme xanthine oxidase.

A study performed by Miller et al. to identify substrate mimics as competitive binders of subunit A of ricin toxin, a plant-derived *N*-glycosidase with significant structural similarity to StxA1, identified pterins and guanine-based compounds as competitive inhibitors of ricin and StxA1.[267] While the main focus of the study was the identification of ricin inhibitors, pteroic acid, 8-methyl-9-oxoguanine, 7-deazaguanine, and 8-methyl-7-deazaguanine were shown to have IC_{50} values against StxA1 between 0.6 and 4.3 mM (Figure 9). In another study

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initiated primarily to identify ricin inhibitors, Wahome and colleagues modified the luciferase-based in situ HTS reported by Saenz and colleagues[101] to screen 81 328 compounds.[268] A secondary in vitro screen of the initial hits that inhibited ricin toxin identified three compounds with *N*-glycosidase inhibitory activity against both ricin toxin and StxA1. The three inhibitors had IC_{50} values between 40 and 500 μM, with 2-[4-[3-(4-fluorophenyl)-1-methylenepropyl]-2-naphthalenyl]-furan (2399297) showing the greatest potency against ricin and StxA1 (Figure 9). The other two compounds were 9*H*-*N*-(2-furanylmethyl)-fluorene-2-sulfonamide and a triazole based thioacetamide. While the weak potency of the inhibitors identified in both of these studies likely precludes the use of these compounds in animal models, they showed that inhibitors of ricin toxin could also inhibit Shiga toxins. It is likely that other reports of inhibitors of ricin toxin will serve as sources of potential leads against Stx due to their similarity.[269,270]

3.11.2. STEC: Inhibitors of Stx2. This section will focus on antivirulence agents targeting Stx2, with reference to small-molecule inhibitors that target both Stx/Stx1 and Stx2 in section 3.11.1. While Stx and Stx1 only differ by one amino acid, the sequence similarity between Stx/Stx1 and Stx2 is only 56%, and in vitro assays report differences in cytotoxicity, with Stx2 having greater cytotoxic effects on human glomerular endothelial cells.[237]

3.11.2.1. STEC: Host Cell Receptor Mimics. In two very similar studies, polymers containing Gb_3-like trisaccharides Gal(α1−4)Gal(β1−4)Glc(β1−*O*-ceramide) were tested for their ability to inhibit Stx2 in vivo when administrated intravenously or orally in mice.[271−273] To develop an injectable therapeutic, these saccharides were assembled onto carbosilane polymers in multiple different orientations and given the name SUPER TWIG (Figure 10).[271,273] On a per trisaccharide basis, SUPER TWIGs containing six saccharide units (SUPER TWIG (1)6) or more were sufficient to occupy the multiple binding sites of the B pentamer and inhibit binding of Stx1 and Stx2 to Vero cells.[271] In a lethal mouse model of *E. coli* O157:H7 infection, IV administration of 50 μg/g SUPER TWIG (1)6 twice a day for 4 days, beginning 3 days after challenge, led to survival of 6/7 mice for 40 days without neurologic symptoms. Administration of SUPER TWIG (1)6 led to a decrease in ^{125}I-Stx2 in the kidney and brain and an increase of toxin in the liver and spleen, though levels were lower than the accumulation of free ^{125}I, leading the authors to hypothesize that Stx2 binding to SUPER TWIG (1)6 resulted in macrophage degradation. Linear acrylamide polymers were also used to test the optimal density of sugar molecules and to develop orally available lead molecules.[272] The authors found that the most densely packed polymer, Gb_3 polymer 1:0, was the most potent in protecting Stx2-mediated cytotoxicity in vitro, as well as in the lethal mouse model of *E. coli* O157:H7 infection. Other groups have built on this idea and tested additional backbones of comparable trisaccharides such as chitosan[274] and carbosilanes (Figure 10).[275]

The same developers of SUPER TWIG then adapted the concept of multivalency to peptides to identify novel inhibitors of Stx2.[276,277] Using the recombinant Stx2 B subunit, multiple rounds of screening with four randomized peptides covalently attached to a polylysine core were performed.[276] The initial screen of tetravalent peptides revealed a strong preference for Arg at P1 and Asn at P3, as well as a general preference for basic residues at all positions. Using these preferences for Arg and Asn in the second round of screening, an optimized peptide PPP-tet, $(MA\text{-}PPPRRRR\text{-}AU)_4$-3Lys, was isolated with a K_D for the monomer peptide of 0.50 μM against Stx2 (Figure 10). Confocal imaging and radiolabeled Stx2 indicated that PPP-tet worked by targeting Stx2 to acidified endosomes, leading to its degradation. Furthermore, using PPP-tet as a tool to study toxin internalization, the authors proposed a model of toxin internalization in which binding site 1 on the B subunit is more important than site 2 for toxin binding to Gb_3, whereas site 3 is important for proper localization of toxin from the Golgi apparatus to the ER. Finally, PPP-tet and the GI-stable acetylated version, Ac-PPP-tet, protected mice in an in vivo lethal mouse model of *E. coli* O157:H7 after established infection (Figure 10). A similar study using Ac-PPP-tet further confirmed the mechanism of action of these multivalent peptides.[277] In juvenile baboons given a lethal dose of Stx2 in which they develop symptoms of HUS, a single IV administration of 5 mg/kg Ac-PPP-tet (called tetravalent peptide, or TVP) given simultaneously with Stx2, as well as 5 mg/kg given IV 6 h after Stx2 challenge followed by four supplement 1 mg/kg doses of TVP on days 1−4, led to 100% survival (3/3) in both treatment groups.[278] Symptoms of acute kidney injury characteristic of HUS—decreased urine output, elevated blood urea nitrogen (BUN), elevated creatinine, and thrombocytopenia—were delayed or mitigated with TVP treatment when monitored over 28 days. However, the anemia characteristic of HUS was not mitigated by TVP treatment. The Nishikawa group further used the same multivalent peptide screen to optimize the peptide sequence and identify peptides capable of binding both Stx1 and Stx2.[279] The peptide sequence $(MA\text{-}MMARRRR\text{-}AU)_4$-3Lys (MMA-tet) was identified that bound the B subunit of Stx1 and Stx2, and was found to be a better inhibitor of Stx2 than PPP-tet. The authors hypothesize that MMA-tet inhibited translocation of the A subunit from the ER to the cytosol, but the exact mechanism remains to be elucidated.

This section highlighted the antivirulence agents developed against Shiga and Shiga-like toxins. Interestingly, most, if not all, studies focused on the treatment of STEC, not *S. dysenteriae* type 1, a significant worldwide source of epidemic dysentery and a serious health threat in terms of antibiotic resistance.[6] Future work should focus on developing antivirulence factors to neutralize Stx caused by *S. dysenteriae* type 1 with drug properties that enable long-term storage and distribution in infrastructure-poor areas where dysentery epidemics typically occur. Similarly, many of the small-molecule inhibitors of Stx focused on Gb_3 mimics that inhibit toxin binding. There is still significant room for the development of inhibitors of StxA *N*-glycosidase activity. Finally, the clinical trials initiated for the treatment of STEC and HUS highlight the difficulty of trial design for antivirulence agents. Crucial factors of design that may have significantly impacted the outcome of the trials highlighted above include: (1) optimal time to initiate treatment for maximal toxin neutralization, in terms of when toxin is released during infection and according to the mechanism of action, pharmacokinetic, and pharmacodynamic properties of the antivirulence agent, and (2) the methodology and tests used to confirm the infectious etiology.

4. CONCLUSIONS AND FUTURE DIRECTIONS

Our continued success in treating infectious disease will increasingly rely on the judicious use of antibiotics and development of novel agents, including antivirulence therapies. To slow the emergence of antibiotic-resistant bacteria, we must

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call on physicians, as well as engage the public, to reduce bacterial resistance by writing prescriptions for antibiotics only when medically indicated. The large number of antivirulence agents highlighted in this review exemplifies a changing paradigm in the treatment of bacterial pathogens, shifting from the use of antibiotics to more tailored therapies. Likely, treatment of bacterial infections will capitalize on combination therapies of antibiotics to decrease bacterial burden and antivirulence agents to selectively target the pathogen and protect from damage to host tissues.

The development and use of antivirulence agents for the treatment of bacterial infections has a number of advantages. First, targeting virulence greatly increases both the number and diversity of drug targets, which will allow for the development of many novel classes of agents with diverse chemical structures to treat bacterial disease. This is essential, as the number of novel targets inhibited by antibiotics is dwindling,[280] and there is some speculation that many of the natural products that are antibiotic in nature have already been discovered.[281] Additionally, since virulence targets are not essential for bacterial survival, treatment with antivirulence agents may not induce as much selective pressure on the bacterium to develop resistance as the targets inhibited by antibiotics, such as protein synthesis machinery. Another benefit of treating bacterial infections with antitoxin agents is the reduced morbidity and damage to host tissues caused by decreased toxin function. The decrease in host cell damage and symptomatic relief, in turn, would allow time for the immune system or host microbiome to fight off the bacterial infection, naturally clearing it without the use of antibiotics. Finally, these antivirulence approaches are highly selective for the bacterial virulence factor of interest and do not cause collateral damage to the host microbiome seen with antibiotic use. Therefore, the use of antivirulence agents, both as monotherapies and in combination with antibiotics, has the potential to limit the ability of bacteria to develop resistance by diversifying both the classes of compounds used and by using compounds with different bacterial targets, as well as by reducing the number of antibiotics needed to treat bacterial infections.

The small-molecule inhibitors highlighted in this review target a large number of novel bacterial enzymes to fight infections, but fit into a few categories of inhibitors. Largely employed against *V. cholerae*, the first class of antivirulence agents target transcription of toxins, inhibiting their transcription and translation.[86−88] These compounds have the benefit of targeting the bacterium instead of the host, and therefore could be targeted to the bacterial niche with less systemic delivery to the host. However, because they act in the bacterium, resistance mutations may occur more readily, and these compounds may be less efficacious later in infection. Another group of antivirulence agents inhibit receptor binding by the toxin. Compounds that inhibit receptor binding do not need to be cell-permeant, a significant hurdle in the development of many drug-like compounds. However, because these compounds act early in the toxin internalization process, they may be less efficacious later in the course of infection. A third class of antivirulence agents inhibits toxin internalization, trafficking, or activation. Because all toxins must be internalized by host cells to exert their cytotoxic effects in the host cytosol, this is the most universal step for toxins. Therefore, many of the compounds that inhibit one of these steps also inhibit cytotoxicity by more than one bacterial toxin and are good candidates to become general antivirulence agents for multiple bacterial infections. On the other hand, many of these agents exert their effects by inhibiting endosomal acidification, which could lead to host toxicity due to the importance of this pathway for physiologic host cell trafficking. For treatment of serious bacterial infections, such as anthrax or botulism, or for short courses of treatment, limited adverse effects may be tolerated on a case-by-case basis. Finally, many antivirulence compounds target the catalytic activity of the toxic domains. While some of these compounds display cross-reactivity with other toxins of the same mechanism, these are typically the most specific compounds for the bacterial pathogen. This is beneficial for preserving the microbiome, but may prove challenging to justify the cost of development through clinical trials for rare infections, such as anthrax, where few prescriptions are likely to be sold.

While significant research efforts have identified many potential antivirulence therapeutic leads, no small-molecule antivirulence agent has successfully become a licensed therapy. Many hurdles remain before an antivirulence agent will make it through clinical trials. As highlighted in this review, there are significant challenges in designing relevant in vitro assays, in vivo animal models, and human clinical trials to test the efficacy of antivirulence compounds. Since traditional in vitro assays to identify novel compounds to treat bacterial infections typically rely on co-incubation of the compound with the bacteria and scoring for bactericidal or bacteriostatic properties, these assays cannot be used to identify antivirulence agents that do not kill the bacterium. Instead, it is necessary to design screening methodologies specific to each toxin to identify lead compounds. Further, the traditional readouts such as minimal inhibitory concentration (MIC) are irrelevant in the context of antivirulence agents. After identification of a promising candidate in vitro, in vivo animal models also present a challenge. Many of the animal models used are intoxication models, in which purified toxin is injected into the animal. These intoxication models, therefore, are not accurate representations of in vivo infection and may be inadequate for some antivirulence compounds, depending on their mechanism of action. Further, some in vivo infection models are more accurate measures of bactericidal properties, and are therefore inaccurate representations of human infection. For example, the classical model of *C. difficile* infection makes use of hamsters, where, unlike humans, infection is uniformly fatal.[282] Therefore, this is not a clinically relevant model for antivirulence agents, as most humans do not die of *C. difficile* infection.[283] Human clinical trial design suffers from the same challenges of adapting classical trial design to antivirulence agents. Finally, for some rare infections, such as anthrax, it is difficult to test compounds in randomized clinical trials due to the very small number and sporadic nature of cases.

Practically, the success of these therapies in the clinic will rely on a detailed understanding of the mechanism of bacterial pathogenesis and the mechanism of inhibitor action to be able to effectively treat patients at the correct time for maximal effect. For example, in the case of antivirulence agents targeting toxin receptor binding, therapies may only be effective early in infection, while antivirulence agents targeting the toxin catalytic site are likely to be more effective after toxins have been internalized. Because many antivirulence factors will only treat one bacterial pathogen, parallel efforts to develop rapid and sensitive diagnostics that can identify the bacterial pathogen are necessary to begin treatment quickly. Among other approaches, there is the potential of chemical tools developed from inhibitor

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scaffolds, such as activity-based probes, to be used as rapid and sensitive in vitro diagnostics as well as for treatment of infection.

Finally, the pharmaceutical industry may lack incentives to develop antivirulence strategies due to their narrow range of use and short treatment courses, making it difficult to recoup investment costs for development. To address this issue with antibiotics, the Generating Antibiotic Incentives Now (GAIN) Act was signed into law in July 2012 to allow pharmaceutical companies developing novel antibiotics an expedited review process and increased exclusivity in the market after approval.[22] To similarly encourage the development of antivirulence agents, the GAIN Act should also apply to these compounds, as they will decrease antibiotic use and therefore reduce the development of bacterial resistance.

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Notes

The authors declare no competing financial interest.

Biographies

Megan Garland graduated summa cum laude from Boston University in 2012 with a bachelor of arts in chemistry with a specialization in biochemistry (with research distinction), and a bachelor of arts in neuroscience. Her undergraduate research with Dr. Karen Allen focused on characterization of enzymes in the haloalkanoate dehalogenase (HAD) superfamily via X-ray crystallography. As an M.D./Ph.D. candidate at Stanford, Megan spent two years working part-time in the laboratory of Dr. Matthew Bogyo while attending medical school before joining the lab full time in June 2014. Her thesis work in the Bogyo lab is focused on nonantibiotic strategies to study bacterial pathogenesis and treat bacterial infections.

Sebastian Loscher received his diploma in chemistry from the University of Stuttgart in 2010. His major focus was the organic synthesis of novel bioactive compounds, carried out with Prof. Peter H. Seeberger during an internship at the ETH in Zurich as well as for his diploma thesis in the lab of Dr. Terry Butters, Glycobiology Institute at Oxford University, U.K. Sebastian moved on to the field of total synthesis and received his Ph.D. at the University of Bayreuth in Germany in the lab of Prof. Rainer Schobert in 2015. He successfully synthesized members of naturally occurring glycotetramates for the first time ("Total synthesis of naturally occurring glycosylated tetramic acids"). In October 2015, he joined the lab of Prof. Matthew Bogyo as a postdoctoral fellow. His work there is focused on the synthesis of bioactive organoselenium compounds and other inhibitors for bacterial toxins.

Matthew Bogyo is a professor of pathology and microbiology and immunology at Stanford University. He received his bachelor's degree in chemistry from Bates College in 1993 and a doctorate in chemistry from Massachusetts Institute of Technology in 1997. He carried out a short postdoctoral fellowship at Harvard Medical School in 1998. Dr. Bogyo established an independent scientific career as a Faculty Fellow at the University of California, San Francisco, in 1998 where he supervised a small laboratory of postdoctoral fellows and students. In 2001, Dr. Bogyo was hired to establish and direct the Chemical Proteomics Department at Celera Genomics focused on applying small molecule probes to the field of drug discovery. Dr. Bogyo then joined the Department of Pathology at Stanford University in July 2003 and was promoted to associate professor in 2009 and to full professor in 2013. His laboratory works on the development of new chemical probe technologies that are applied to study the role of proteases in complex biological pathways associated with human disease. Dr. Bogyo has published over 200 primary research publications and currently serves on the editorial boards of several prominent research journals and is a consultant for several biotechnology and pharmaceutical companies in the Bay Area.

LIST OF ABBREVIATIONS

4-APP	4-aminopyrazolol[3,4-*d*]pyrimidine
ABH	α/β hydrolase domain
ABP	activity-based probe
Ac	acetyl
ACD	actin cross-linking domain
AOMK	acyloxymethyl ketone
APE	apple polyphenol extract
AVA	Anthrax Vaccine Adsorbed, also called BioThrax
BABIM	bis(5-amidino-2-benzimidazolyl)methane
BIG-IV	botulinum immune globulin, also called BabyBIG
BoNT	botulinum neurotoxin
BTMTs	benzothiazole-4-methylthiophenes
BTTs	benzothiazole thiophenes
BUN	blood urea nitrogen
cAMP	cyclic adenosine monophosphate
Cbz	carboxybenzyl
CDC	Centers for Disease Control and Prevention
CDI	*C. difficile* infection
CFTR	cystic fibrosis transmembrane conductance regulator
CPD	cysteine protease domain
CPI	cyclic peptide inhibitor
CRE	carbapenem-resistant Enterobacteriaceae
CROP	combined repetitive oligopeptide
CT	cholera toxin, also referred to as choleragen and CTX
CTA	A subunit of cholera toxin
CTA1	A1 fragment of cholera toxin
DCCHD	dicarboxy cyclohexanediol
DT	diphtheria toxin
DTA	A subunit of diphtheria toxin
eEF2	eukaryotic elongation factor 2
EF	edema factor
EGCG	(−)-epigallocatechin-3-gallate
ER	endoplasmic reticulum
ESBL	extended spectrum β-lactamase
ET	edema toxin
ETA	exotoxin A
ETEC	enterotoxigenic *E. coli*
FDA	U.S. Food and Drug Administration
FP HTS	fluorescence polarization high-throughput screen
FRET	Förster resonance energy transfer
GAIN Act	Generating Antibiotic Incentives Now Act
Gal	galactose
$G_{\alpha s}$	G_s α-subunit
Gb_3	Gal(α1−4)Gal(β1−4)Glc(β1−*O*-ceramide)
Glc	glucose
GM1	Gal(β1−3)GalNAc(β1−4)[NeuAc(α2−3)]Gal-(β1−4)Glc(β1−*O*-ceramide)
GTD	glucosyltransferase domain

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HBV	hepatitis B virus
HC	heavy chain
H_C	C-terminus of the heavy chain
H_N	N-terminus of the heavy chain
H-NS	histone-like nucleoid structuring protein
$H_2NPr\beta CD$	per-6-(3-aminopropylthio)-β-cyclodextrin
Hpa	hydroxy-phenyl acetyl
HPLC	high-performance liquid chromatography
HRCECs	human renal cortical epithelial cells
HTS	high-throughput screen
HUS	hemolytic uremic syndrome
IND	investigational new drug
IP_6	1D-*myo*-inositol hexakisphosphate
LC	light chain
LF	lethal factor
LF-β-lac	LF-β-lactamase
LT	heat-labile enterotoxin (from *E. coli*) or lethal toxin (from *B. anthracis*, also abbreviated as LeT or LeTx)
MALDI	matrix-assisted laser desorption ionization
MANT	*N*-methylanthraniloyl
MAP	multiple antigen peptide
MAPKKs	mitogen-activated protein kinase kinases, also abbreviated as MEKs
MARTX	multifunctional-autoprocessing-repeats-in-toxin
MDT	3-(methylthio)-1,4-diphenyl-1*H*-1,2,4-triazolium bromide
MDR	multidrug-resistant
MIC	minimal inhibitory concentration
MMPs	matrix metalloproteases
MNPG	*m*-nitrophenyl-α-D-galactoside
NAP	1,8-naphthalimide
NBTI	nitrobenzylthioinosine
NCI	National Cancer Institute
NTCD-M3	nontoxigenic *C. difficile* strain M3, also known as VP20621
OLDA	*N*-oleoyl-dopamine
PA	protective antigen
PaLoC	pathogenicity locus
PARPs	eukaryotic poly(ADP-ribose) polymerase enzymes
PBT	pentavalent (ABCDE) botulinum toxoid
PGG	1,2,3,4,6-penta-*O*-galloyl-β-*D*-glucose
PI3K	phosphatidylinositide 3′-OH kinase
PT	pertussis toxin
PTA	A subunit of pertussis toxin
PVI	polyvalent inhibitor
qHTS	quantitative high-throughput screen
RID	Rho-inactivating domain
RG	rhubarb galloyl
SAMs	self-assembled monolayers
SAP	serum amyloid P component
SAR	structure−activity relationship
SNAP-25	synaptosome associated protein 25
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
ST	heat-stabile enterotoxin
STEC	Shiga toxin producing *E. coli*
STP	spontaneously translocating peptide
Stx	Shiga toxin
Stx1	Shiga-like toxin 1
Stx2	Shiga-like toxin 2
TAMRA	carboxytetramethylrhodamine
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
TCP	toxin coregulated pilus
TeNT	tetanus toxin, also known as tetanospasmin
TscH	*C. sordellii* hemorrhagic toxin
TscL	*C. sordellii* lethal toxin
TUDCA	tauroursodeoxycholic acid
TVP	tetravalent peptide
WHO	World Health Organization

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