New Therapeutic Developments against Shiga Toxin-Producing Escherichia coli

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ABSTRACT Shiga toxin (Stx)-producing Escherichia coli (STEC) is an etiologic agent of bloody diarrhea. A serious sequela of disease, the hemolytic uremic syndrome (HUS) may arise in up to 25% of patients. The development of HUS after STEC infection is linked to the presence of Stx. STEC strains may produce one or more Stxs, and the Stxs come in two major immunological groups, Stx1 and Stx2. A multitude of possible therapeutics designed to inhibit the actions of the Stxs have been developed over the past 30 years. Such therapeutics are important because antibiotic treatment of STEC infections is contraindicated due to an increased potential for development of HUS. The reason for the increased risk of HUS after antibiotic treatment is likely because certain antibiotics induce expression of the Stxs, which are generally associated with lysogenic bacteriophages. There are a few potential therapeutics that either try to kill STEC without inducing Stx expression or target gene expression within STEC. However, the vast majority of the treatments under development are designed to limit Stx receptor generation or to prevent toxin binding, trafficking, processing, or activity within the cell. The potential therapies described in this review include some that have only been tested in vitro and several that demonstrate efficacy in animals. The therapeutics that are currently the furthest along in development (completed phase I and II trials) are monoclonal antibodies directed against Stx1 and Stx2.

BACKGROUND Shiga toxin (Stx)-producing Escherichia coli (STEC) colonizes the intestine and causes hemorrhagic colitis. STEC encodes a variety of colonization factors, but a significant subset of STEC, the enterohemorrhagic E. coli (EHEC) strains, have the locus of enterocyte effacement (LEE), the products of which allow the bacteria to intimately adhere to and form attaching and effacing lesions on intestinal tissue. The O157:H7 strains, which are responsible for the majority of large outbreaks due to STEC infection, are members of the EHEC group. All STEC strains make one or more Stxs; these pathogens may produce two immunologically distinct but highly similar Stxs, Stx1 and Stx2. These toxins are briefly described in the section on therapeutics targeted to the Stxs.

Some individuals infected with STEC manifest a serious sequela called hemolytic uremic syndrome (HUS), a thrombotic microangiopathy defined by the presence of hemolytic anemia, thrombocytopenia, and renal failure. The initial insult leading to the development of the thrombotic microangiopathy is damage by the Stx(s) to vascular endothelial cells that express the toxin receptor, globotriaosylceramide (Gb3). The Stx-mediated injury to endothelial cells initiates a cascade of events that lead to the activation of platelets and the formation of thrombi in the small vessels of the kidney and sometimes
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the central nervous system. Prevention of HUS is important as HUS can lead to death or long-term consequences such as hypertension and renal disease (1–3). Because STEC strains are intestinal bacterial pathogens, the inclination by physicians is to treat with antibiotics to eliminate the organisms from the gut. However, several studies show that treatment of STEC-infected patients with certain antibiotics may lead to an increase in HUS (antibiotic use is discussed further in the next section). Therefore, the focus for therapeutics against STEC and HUS has been to find (i) compounds that act at the level of the bacterium but do not cause an increase in Stx production; (ii) receptor mimics or other molecules that alter trafficking of the toxin or the cellular response to the toxin; (iii) antibodies directed against the Stxs; or (iv) therapies to prevent or treat the HUS disease process. The therapies discussed in this article are listed in Table 1.

THE DIFFICULTIES WITH ANTIBiotic USE FOR STEC INFECTIONS

In the United States, antibiotics are not recommended for treatment of STEC infections because of the increased risk for the development of HUS (4, 5). However, even though antibiotics are contraindicated for those with STEC infection, a recent study at FoodNet sites found that antibiotics are commonly used in those with proven O157 infection (6). The issue of the use of antibiotics to treat STEC infections is confounded by a number of factors: (i) some, but not all, antibiotics at sublethal doses increase the expression of Stxs by inducing the lysogenic phage that encodes the toxin genes (see diagram in Fig. 1) (7); (ii) treatment of STEC with a lethal dose of antibiotics may cause release of a large bolus of toxin at one time as the bacteria die—a result that would be bad for a patient; (iii) in some STEC strains, the Stxs are either chromosomally encoded or are associated with defective bacteriophages, and thus antibiotic treatment may not alter Stx expression; (iv) antibiotics may be used only in the most ill patients, a fact that may skew the apparent risk for HUS development; and (v) published studies on the risk of HUS associated with antibiotic treatment used different treatments administered at different stages in the disease process. In addition, although several studies demonstrate an increased risk for HUS after treatment with antibiotics (8, 9), others do not find an increased risk (10). Conversely, several studies indicate that the use of β-lactam antibiotics, particularly in the first 2 to 3 days of illness, and perhaps associated with age <13 years, is correlated with increased HUS risk (11). In contrast, many people were treated with fosfomycin during a large O157:H7 outbreak in Japan without an apparent increase in HUS, and perhaps even a protective effect if the antibiotic was given within the first 2 days of illness (12). However, the low overall HUS rate in the Sakai outbreak (13) suggests that the causative strain may (fortunately) have had reduced virulence compared with O157 strains from other outbreaks, and, as such, would make that outbreak a poor platform on which to make generalized decisions about treatment with antibiotics.

During the O104:H4 outbreak in Germany in 2011 caused by an unusual enteroaggregative E. coli (EAEC) strain that makes Stx2, antibiotics were used in many patients, some of whom were given up to three antibiotics at various times after disease onset (14). Because of the vastly different protocols used to treat patients during the outbreak in Germany, it is difficult to make generalized conclusions about antibiotic use based on that outbreak; however, overall there was not definitive evidence of a benefit due to antibiotic treatment. Although one surprising study asserted ciprofloxacin treatment reduced the HUS incidence in patients, the number of treated patients was small (n = 5), and in two of those patients HUS did develop (15). Furthermore, in several patients from the O104:H4 outbreak HUS was reported to develop after ciprofloxacin or metronidazole treatment (16, 17). We consider the use of ciprofloxacin to be especially dangerous because of the evidence that ciprofloxacin increases Stx expression in the EAEC O104:H4 strain (18) and STEC O157 strains. Finally, one unique aspect of the outbreak in Germany was the use of azithromycin to reduce shedding of O104:H4 in patients who appeared to be long-term carriers (19).

Animal studies also give contradictory information about the use of antibiotics for the treatment of STEC infections. In two studies fosfomycin reduced colonization and mortality from STEC infection in mice (20, 21), but another study did not show a protective effect by that antibiotic (22). In a mouse model in which the mice are starved for protein calories, the use of trimethoprim-sulfamethoxazole was detrimental when given between days 3 and 5 post infection, whereas the same antibiotic as well as ampicillin and fosfomycin were protective when given anywhere from days 1 to 5 after infection (23). Nevertheless, we should note that the use of ampicillin to treat an infection with an organism (Shigella dysenteriae or the Stx2+ EAEC O104:H4, respectively) resistant to that antibiotic appears to be detrimental to humans and mice (24, 25).
Overall, the current data do not support the use of antibiotics for the treatment of STEC infection in humans, and the majority of the evidence indicates that antibiotic treatment is potentially detrimental. The empirical use of antibiotics could be dangerous, since some of these treatments could increase the risk of HUS. Furthermore, since there is no clear demonstrable benefit to the use of antibiotics in STEC-infected patients, and because antibiotic use itself may pose possible risk to the patient (allergy or other risks), we believe antibiotics should not be used to treat STEC infections.

**COMPOUNDS DIRECTED TOWARD STEC**

**Pyocin**
A novel strategy to kill O157 strains based on a modified pyocin (a bactericidal protein, similar to bacteriophage tail fibers, made by *Pseudomonas aeruginosa* that usually targets other *P. aeruginosa* cells) that consists of the R2 tail fiber fused to a phage spike protein specific for O157 was found to kill O157 strains without increasing expression of Stx (26). Subsequent tests demonstrated that a slightly modified version of the pyocin, AvR2-V10.3, given 3 h after infection and once daily for the next 2 days to infant rabbits infected with O157:H7 strain EDL933 reduced colonization and prevented diarrhea at the highest dose of pyocin administered (27). When the modified pyocin was given to EDL933-infected infant rabbits that exhibited diarrhea, the amount of loose stool decreased relative to the control animals given buffer alone and the numbers of EDL933 organisms decreased in the intestines and stools. One problem with the pyocin approach is that it is specific for serogroup, so other pyocins would have to be developed for non-O157 STEC strains.

**A Small Molecule and a Divalent Cation**
Another alternative strategy to antibiotics directed toward the bacterium uses a small molecule, LED209, which inhibits the activity of the QseC sensor that is involved in regulating some of the proteins involved in EHEC adherence and, indirectly, Stx2 expression. Although LED209 inhibited the formation of attaching and effacing lesions by EHEC on HeLa cells, the compound was not effective in reducing colonization or disease in infant rabbits (28). The reason for LED209 failing to protect in the rabbits may be that the concentration of the compound was not high enough at the site of EHEC colonization in the gut.

Similar to LED209, treatment of STEC infection with the divalent cation zinc reduces adherence to a HeLa cell monolayer, though through a different mechanism, perhaps due to decreased expression of *E. coli*-secreted proteins EspA and EspB (29). In addition to a reduction in adherence to cells, zinc treatment also appears to decrease expression of the *stx* genes. The potential therapeutic effect of zinc was tested in a rabbit ileal loop model, in which rabbit enteropathogenic *E. coli* strains transduced with a bacteriophage that encodes *stx*1 or *stx*2 were inoculated into the loop in the presence or absence of zinc. The presence of 1 mM of zinc reduced the amount of toxin found in the loops, decreased adherence, and lessened histological damage. The next avenue to explore for zinc is to determine if the cation could prevent disease or mortality in an oral infection model.

**THERAPEUTICS THAT INTERFERE WITH TOXIN BINDING, UPTAKE, TRAFFICKING, OR FUNCTION**

Stx1 and Stx2 are the key STEC virulence factors that lead to the development of HUS. Therefore, therapeutics that impede toxin binding, uptake, trafficking, or function are strong contenders for treatment of STEC infections. Stx1 and Stx2 share about 56% homology at the amino acid level but are immunologically distinct. Structurally, the toxins consist of five identical B subunits and a single A subunit. The B pentamer binds to the cellular receptor, Gb3. The A subunit contains the enzymatic function of the toxin and removes an adenine residue from the 28S rRNA, an action that destroys ribosome function. After binding to Gb3, the toxin receptor complex is taken up by clathrin-dependent and -independent mechanisms. The toxin then traffics in a retrograde direction from the endosome to the Golgi apparatus to the endoplasmic reticulum (ER). The A subunit of the toxin can be nicked within the Golgi body such that the enzymatic function (within A1) is separated by a disulfide bond from the A2 peptide that links A1 to the B pentamer. The nicked toxin traffics next to the ER where the disulfide bond between A1 and A2 is reduced. The A1 subunit then enters the cytoplasm and targets the ribosome. The damage to the ribosome halts protein synthesis and can lead to a ribotoxic stress response and apoptosis (see review in reference 30). A model of Stx trafficking is shown in Fig. 2.

Stx1 and Stx2 are the major toxin types produced by STEC that cause human disease. There are subtypes of both Stx1 and Stx2, however, and toxin nomenclature...
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| **Antitoxin antibodies**  
(B, U, T, or E) | **Polyclonal anti-Stx2**  
casStx1 (human/mouse chimeric  
version of 13C4) | **Neutralize Stx2**  
Block Stx1 binding (B subunit) | **Gnotobiotic pigs**  
Vero cells; Stx1-intoxicated mice;  
phase I and II trials | 62, 63, 68, 70 |
| --- | --- | --- | --- | --- |
| **caStx2 (anti-A subunit,  
human/mouse chimeric  
of 11E10)** | **Alters intracellular trafficking and  
inhibits enzymatic function** | **Vero cells; streptomycin-treated mice;  
phase I and II trials** | 63, 68–70 |
| **Anti-Stx1 (5-5B)** | **Neutralize Stx1 (B subunit)** | **Ramos cells** | 71 |
| **Urtoxazumab (also called  
TMA-15; humanized  
version of VTm1.1)** | **Neutralize Stx2 (B subunit)** | **Human renal  
adencarcinoma cells; Stx2-intoxicated mice;  
B2F1-infected streptomycin-treated mice;  
phase I trial** | 73–75 |
| **Anti-Stx1 monoclonals** | **Neutralize Stx1 (most are anti-B subunit)** | **HeLa cells; Stx1-intoxicated mice** | 76 |
| **Anti-Stx2 (5C12)** | **Neutralize Stx2 (A subunit)** | **HeLa cells; Stx2-intoxicated mice;  
B2F1-infected streptomycin-treated mice;  
O157-infected gnotobiotic piglets** | 77, 78 |
| **Toxin transport (U or T)** | **Exo2** | **Blocks transport at the level of the early  
endosome/trans-Golgi interface** | **Vero cells** | 82, 83 |
| **Retro-2<sup>cry</sup>** | **Toxin transport** | **HeLa cells** | 85, 86 |
| **Chloroquine** | **Toxin transport** | **HeLa cells** | 87 |
| **Small molecules** | **Toxin transport** | **HeLa cells** | 88 |
| **Eeyarestatin 1** | **Intracellular trafficking** | **Human renal cortical epithelial cells** | 90 |
| **Nitrobenzyl-thioinosine** | **Stx1 trafficking  
(retention in early endosomes)** | **HeLa cells; Stx1-intoxicated BALB/c mice  
(does not protect Stx2-intoxicated mice)** | 92, 93 |
| **Manganese** | **Protects HeLa cells and BALB/c mice from  
Stx1-mediated lethality; blocks StxB1  
 trafficking; does not protect against Stx2  
 trafficking** | **HEP-2 cells** | 94 |
| **Toxin processing (N)** | **Furin inhibitor** | **Enzymatic activity** | **Cell-free reporter assay** | 89 |
| **Toxin function (E)** | **Small molecule** | **Ribotoxic stress response inhibitor** | **HCT-8 cells; infant rabbit** | 98 |
| **Cellular or host response  
to toxin (ribotoxic stress  
response or apoptosis)** | **Imatinib** | **Apoptosis inhibition** | **Rat proximal tubule cells (RPTC)** | 99 |
| **Complement factor C5** | **Eculizumab** | **Anticomplement factor C5** | **STEC-infected patients** | 101, 105, 106 |
was recently updated so that the prototype Stxs are now designated Stx1a and Stx2a. The less specific Stx1 and Stx2 designations are used when the toxin subtype is unknown (31). We use the more general designations for the toxins elsewhere in this review for simplicity. Besides the prototypic Stx2a, two Stx2 subtypes are associated with HUS, Stx2c and Stx2d (32, 33). Stx2c and Stx2d have two amino acid differences in the B subunit as compared to Stx2a, and those changes are responsible for reduced cytotoxicity on Vero cells, though Stx2d is just as toxic to mice as Stx2a (34). Stx2d has two amino acid differences from Stx2a and Stx2c in the A subunit, and those two changes contribute to the capacity of the toxin to exhibit increased toxicity when treated with elastase from intestinal mucus (35), a phenotype indicated with the designation Stx2dact.

Receptor Synthesis Inhibitor: C-9

One step at which to stop Stx is at the level of the toxin receptor. Various laboratories have tried to protect cells or animals from Stxs with compounds that prevent Gb3 synthesis or that bind to the toxin to prevent toxin-receptor interaction. For example, a molecule called C-9 was used in human renal tubular epithelial cells (HRTEC) to prevent the conversion of ceramide to glucosylceramide, an early step in Gb3 synthesis. HRTEC pretreated with C-9 for 24 h exhibited reduced levels of Gb3 and decreased sensitivity to Stx2 (36). In rats treated with C-9 for 2 days before intoxication and for 4 days post intoxication, about 50% protection from infection with bacterial supernatants that contained Stx2 was observed (37). Treated rats also showed lower rises in serum creatinine and urea and reduced renal tubular injury.

Receptor Analogs

The first drug tested in humans intended for the treatment of HUS was SYNSORB Pk, a silicon dioxide compound that contains the trisaccharide component of Gb3 (38, 39). The therapy behind the use of SYNSORB Pk is that the compound would bind up free Stx(s) within the intestines of infected patients and prevent that toxin from binding to the functional receptor so that the toxin could not act either locally or systemically. SYNSORB Pk was shown to neutralize both Stx1 and Stx2 on human renal adenocarcinoma cells (40). Although SYNSORB Pk was tolerated well in the phase I trial (41), the double-blind placebo-controlled trial suggested no difference between treated and placebo groups (39). The reason for the lack of efficacy by SYNSORB Pk in the latter study may be that the patients were enrolled after HUS diagnosis, whereas the best time to neutralize toxin to prevent HUS is most likely before development of this serious sequela.

A number of other Stx receptor analogs in addition to SYNSORB Pk have been developed, such as STARFISH (42), Daisy (43), SUPER TWIG (1/6 (44), Gb3 polymers (45), and Ac-PPPtet (46), as well as a probiotic that displays an Stx binder on its surface (47). STARFISH is a five-“armed” molecule with two receptor mimics at the end of each arm; the compound appears to bind two B pentamers at the same time and neutralizes both Stx1...
and Stx2 in vitro. However, STARFISH has a higher avidity for Stx1 than Stx2 and was only able to protect mice injected with Stx1 but not Stx2 (43). Because of the lack of protective efficacy by STARFISH in the Stx2-injection model, a similar but modified divalent trisaccharide inhibitor called Daisy was synthesized (43). Daisy protects Vero cells and mice from both Stx1 and Stx2, and in a streptomycin-treated mouse model, prevented death due to infection with O91:H21 STEC strain B2F1 in 50% of the animals (43). The SUPER TWIG (1)6 identified by Nishikawa’s group carries six trisaccharides and blocks the binding of both Stx1 and Stx2 to Vero cells, protects mice from Stx2 when administered together with the toxin, and prevents death of O157:H7-infected mice in a protein-calorie-deficient animal model when given intravenously after infection (44). However, one point to note is that SUPER TWIG (1)12, developed in the latter study, neutralized as well as SUPER TWIG (1)6 in vitro but did not protect in vivo, a finding that demonstrates the importance of testing potential therapeutics in an animal model system. To find a receptor analog that could be given orally, the Nishikawa group developed Gb3 polymers that bind to the Stxs with higher affinity than SUPER TWIG (1)6, and those polymers, when given by gavage twice daily in the protein-calorie-deficient mouse model on days 3 to 5 after infection, protect mice from death (45). The strong neutralization effect observed with SUPER TWIG and the Gb3 polymers is due to the fact that the B pentamer of the Stxs contains multiple Gb3-binding sites, so receptor mimics that display multiple copies of the Gb3 trisaccharide bind the toxin tightly. A similar approach as described above was used by another group to display the receptor sugar moiety on chitosan, and they similarly found that the analog neutralizes in vitro and in vivo (48).

According to Nishikawa, however, a drawback to the multiple trisaccharide display approach is the complexity of synthesis (46, 49); therefore, a tetravalent peptide library was screened to find molecules that bind the Stx2
pentamer (49). In that latter screen, tetravalent peptides were identified that form a complex with Stx2 and neutralize the toxin on Vero cells but do not prevent the toxin/peptide moiety from binding to the cell. Rather, the peptide-bound Stx2 was unable to reach the ER. Furthermore, O157-infected protein-calorie-deficient mice gavaged after infection with the peptides were protected from death; the protection appeared to be dose dependent and treatment had to be started by day 3 post infection. Further testing of the optimized tetrapeptide, Ac-PPP-tet (later renamed TVP), demonstrated that the compound inhibits Stx2-mediated fluid accumulation in rabbit ileal loops (46). However, the tetravalent peptide only protects when administered orally and not intravenously in the protein-calorie-deficient mouse model, so TVP efficacy was tested in the baboon model of intoxication (50). Baboons given Stx2 and TVP simultaneously were protected from death, renal injury, and thrombocytopenia, but not anemia. TVP also rescued 75% of animals that received the drug 24 h post intoxication and a supplemental dose on days 2, 3, and 4. Untreated but intoxicated baboons died by day 6. The authors of that study suggest that the advantage of TVP compared to other Stx binders is that the compound is cell-permeable. However, it is unclear how TVP would rescue intoxicated cells since the proposed mechanism for action is for the Stx2-TVP complex to traffic differently within the cell than Stx2 alone, and indeed, in vitro, cells are only protected if the TVP is added to cells at the same time as Stx2. In a recent study, the Nishikawa group identified another tetravalent peptide, MMA-tet, that inhibits both Stx1 and Stx2 on Vero cells at the same time as Stx2. In vitro, cells are only protected if the TVP is added to cells at the same time as Stx2. In a recent study, the Nishikawa group identified another tetravalent peptide, MMA-tet, that inhibits both Stx1 and Stx2 on Vero cells when it is added at the same time as the toxins, and when administered orally, protects protein-calorie-deficient mice infected with STEC strain N-9 (51). The mechanism whereby MMA-tet protects cells and animals is unclear: MMA-tet forms a complex with the Stx1 B subunit but does not appear to alter binding or trafficking of the MMA-tet–StxB1 through the cell, at least as far as the ER. However, MMA-tet pretreatment of Vero cells did prevent protein synthesis inhibition by Stx1, a finding that may indicate that MMA-tet somehow prevents the toxin A1 subunit from reaching the cytoplasm from the ER.

Finally, a novel modification of the receptor analog approach to bind up Stx specifically within the intestine was designed by Paton’s group: the core trisaccharide fromGb3, Galα1-4Galβ1-4Glc-, was displayed on the lipopolysaccharide (LPS) structure of a commensal E. coli to create a probiotic (52). The constructed strain, CWG308:pJCP-Gb3, neutralizes Stx1, Stx2, Stx2c, and Stx2d on Vero cells. In addition, streptomycin-treated mice are protected from infection with either an Stx2- or Stx2d-producer when fed the Galα1-4Galβ1-4Glc-probiotic twice daily. What was not clear in that study was whether the mice would continue to do well once the probiotic was discontinued. However, the probiotic with a receptor-analog approach might be possible as long as the infecting strain did not persist for long-term colonization. Additionally, it may be necessary to monitor shedding of the infecting strain over time. CWG308:pJCP-Gb3 was found to be effective even when killed prior to administration; however, the formaldehyde-treated probiotic had to be administered three times daily for complete protection (53). To develop a probiotic that might be able to be used in humans, a K-12 strain that expressed the same Galα1-4Galβ1-4Glc- epitope on its LPS was created (54). In that strain, additional mutations were incorporated so that no antibiotic resistance markers were needed for plasmid maintenance. The K-12 probiotic that displays Galα1-4Galβ1-4Glc- neutralizes both in vitro and in vivo (54).

Human Serum Amyloid Component P (HuSAP) Reports that there is an Stx2-neutralizing component in normal human serum that was not immunoglobulin were published in 1993 (55, 56). Later, HuSAP was identified as the protein from plasma that can neutralize Stx2, but not Stx1 (57). HuSAP administered intravenously protects BALB/c mice given Stx2 1 h later (58). Another group showed similar results, though they administered the HuSAP twice daily intraperitoneally and the Stx2 by the subcutaneous route (59). This group then challenged transgenic C57Bl/6 mice that express HuSAP with two 50% lethal doses of Stx2. The HuSAP-transgenic mice survived nearly twice as long as the control mice in that study. The reason that the HuSAP-injected BALB/c mice survived longer than the HuSAP-transgenic mice was hypothesized to be because the circulating levels of HuSAP were higher in the BALB/c mice injected with HuSAP than in the transgenic animals. If, however, the HuSAP-transgenic mice were injected with a combination of LPS and Stx2, the animals were not protected, even though the LPS did not prevent HuSAP-Stx2 interaction or the neutralization by HuSAP of Stx2 for Vero cells in vitro (60). Exactly how HuSAP binds Stx2 is not clear; Marcato et al. reported that the interaction requires both toxin A and B subunits and cannot be competed with Daisy (61). The latter finding suggests that HuSAP does not bind within the Gb3-binding sites on the B pentamer.
**ANTIBODIES TO THE Stxs**

Because the Stxs are the primary factors responsible for the development of HUS, neutralization of the toxins is a critical therapeutic approach for STEC infections. Polyclonal antiserum against Stx2 protects gnotobiotic piglets from infection by O157:H7 strain 86-24 (62). Furthermore, monoclonal antibodies to the toxins are protective in animal models, and two groups have taken such therapeutics into phase II safety trials, as detailed below.

Monoclonal antibodies specific for the Stx1 B subunit (13C4) and the Stx2 A subunit (11E10) were developed in the O’Brien laboratory in the mid to late 1980s (63, 64). Those antibodies neutralize the toxins on Vero cells (65, 66), and the Stx2 monoclonal antibody is protective in mice (67). The epitopes for both antibodies have been mapped (65, 66); of note, although 11E10 neutralizes Stx2 in a cell-free protein synthesis assay, the antibody also alters the intracellular localization of the toxin such that the toxin-antibody complex does not reach the cytoplasm (65). Both 11E10 and 13C4 were transformed into human/mouse chimeras by genetic techniques. The “humanized” versions of anti-Stx1 and anti-Stx2 neutralize the toxins on Vero cells and in either a mouse intoxication model (Stx1) or the streptomycin-treated mouse model of infection with strain B2F1 (68). The humanized versions of the antibodies (cuStx1 for 13C4 and cuStx2 for 11E10) were evaluated in phase I (69, 70) and II trials. The preliminary results of the phase II trial indicate that the antibodies were safe and well tolerated in sick children infected with STEC.

Takeda’s group developed monoclonal antibodies that neutralize Stx1 or Stx2 by preventing receptor binding (71, 72). The anti-Stx2 monoclonal was later humanized and named TMA-15. TMA-15 demonstrates Vero cell neutralization and protective efficacy in animal models (73, 74). Antibody TMA-15 was renamed urtoxazumab and evaluated in safety trials in healthy adults and STEC-infected children (75). Urtoxazumab was well tolerated in STEC-infected children, but no efficacy data are yet published.

Finally, Tzipori’s group generated fully humanized neutralizing monoclonal anti-Stx1 and -Stx2 antibodies in transgenic mice (76, 77). The anti-Stx2 monoclonal antibody, 5C12, was developed further, and shown to protect piglets and streptomycin-treated mice from STEC strains that produce Stx2 or Stx2dact, respectively (77, 78). Similarly to 11E10, antibody 5C12 neutralizes Stx2 by altering its intracellular trafficking pattern and also has the capacity to prevent protein synthesis inhibition by Stx2 in a cell-free assay (79, 80). The Tzipori group also evaluated isotype variants and Fab and F(ab′)2 fragments of 5C12 in vitro and in vivo models of efficacy and found that all of the isotype variants demonstrate in vitro and in vivo neutralization but the Fab and F(ab′)2 were only efficacious in the in vitro assay (81). One point of note from the latter study is that the IgG4 variant of 5C12 was the least protective of the isotype variants in vitro, but was as protective in vivo as the best in vitro neutralizer (the IgG3 variant). These latter results indicate once again that in vitro and in vivo neutralization data do not always correlate.

**Nonantibody Inhibitors of Toxin Trafficking or the Cellular Response to Stx**

Brefeldin A (BFA), a fungal toxin that disrupts the Golgi apparatus but also causes tabulation of early endosomes, is a known inhibitor of Stx transport; in fact, the protective effect of BFA against the Stxs helped define the pathway by which Stx is transported through the cell. Because BFA is a global inhibitor of protein transport within the cell, it is not included in Table 1.

Another Golgi disruptor, Exo2, a small molecule that does not inhibit cholera toxin trafficking, prevents Stx from reaching the Golgi apparatus (82). Unfortunately, cell disruptors such as BFA and Exo2 are toxic to cells. Another drawback to molecules such as BFA and Exo2 is that treated cells recover over time, such that incubation of the cells with the inhibitor and the toxin for longer periods reduces the effectiveness of the therapeutic. Exo2 was recently derivatized to generate an inhibitor with lower toxicity that still interferes with Stx trafficking (83). Another small molecule, Retro-2, blocks Stx from reaching the trans-Golgi network (84, 85) and is effective against ricin, a toxin that traffics in the same manner as the Stxs. Recently, a derivative of Retro-2, Retro-2cyc, was identified. Retro-2cyc is 100-fold more active on HeLa cells than Retro-2 (86). However, the drawback to Retro-2cyc and derivatives is that the cells needed to be pretreated with the drug to observe protection from the toxin (84, 86). Another compound known to protect cells from Stx, chloroquine, was also recently shown to permit the toxin to reach the ER (87). Since chloroquine does not inhibit the enzymatic activity of Stx nor degrade the toxin in vitro, the drug likely acts by inhibiting the toxin from exiting the ER.

Further small-molecule screens have identified other compounds that inhibit Stx and ricin transport within Vero cells (88) or the enzymatic activity of both toxins (which have the same mode of action) (89). Another small molecule, eeyarestatin I, which interferes with
intracellular trafficking among cellular compartments, was shown to cause a lag in protein synthesis inhibition in HeLa cells by Stx (90). Finally, nitrobenzylthioinosine was shown in 2002 to inhibit the trafficking of Stx1, trapping the toxin within the early endosome (91); however, no additional information on the use of this compound to protect cells or animals has been published.

A recent report suggests that manganese inhibits intracellular trafficking of the B subunit of Stx (92), a finding that may or may not extend to the holotoxin. However, manganese treatment increased the viability of HeLa cells incubated with Stx1 in that study and protected BALB/c mice injected with 500 ng of Stx1 and injected with daily doses of manganese (at least 10 mg/kg daily injections were required). Whether such levels of manganese would be reasonably achievable in a patient is not clear. In addition, manganese fails to protect HeLa cells from Stx2 intoxication (93), a finding that makes the potential use of manganese less likely as Stx2 is more commonly associated with HUS development than Stx1.

**A COMPOUND THAT INTERFERES WITH TOXIN PROCESSING**

Because the A subunit must be cleaved by a furin- or trypsin-like protease so that the A1 or enzymatically active moiety of the toxin may be separated from the holotoxin, furin inhibitors were tested in a cell-based assay for the capacity to protect HEp-2 cells from Stx (94). Although one of the inhibitors showed moderate inhibition activity against Stx, the inhibition was overcome at higher concentrations of Stx. Furthermore, since the toxin can be cleaved by enzymes in intestinal mucus (35), as well as by intracellular proteases (95), and because Stx mutated at the trypsin-sensitive site can still be cleaved (96, 97), we suspect that it will be difficult to protect animals from Stx with protease inhibitors.

**Therapies That Interfere with the Cellular Response to Stx**

Inhibitors of mitogen-activated protein kinase pathways were used to assess potential protective efficacy against the Stx2-mediated ribotoxic stress response in HCT-8 cells or for oral gavage of Stx2 into infant rabbits (98). The authors observed modest protective capacity by imatinib in HCT-8 cells and against some aspects of Stx2 intoxication in the animals, specifically heterophil infiltration into the intestine. That study indicates that the infant rabbit model of Stx2 gavage may prove useful for the evaluation of therapeutics. However, such a model would be expensive, and determining effective therapeutic doses and the timing for those doses might prove challenging.

A recent paper suggests that an inhibitor of apoptosis, ouabain, protects rat renal proximal tubules from Stx-mediated cell death (99). Kidneys from ouabain-treated mice that were given about four 50% lethal doses of Stx2 showed reduced podocyte depletion as compared to phosphate-buffered saline–treated mice. However, no data were reported on whether the ouabain could protect the mice from Stx2-mediated lethality.

**TREATMENT ONCE HUS IS DIAGNOSED**

What do you do for patients who already have HUS? We leave discussion of specific clinical interventions such as dialysis and apheresis for medical experts. In terms of a therapeutic that may interfere in the etiology of HUS, Lapeyraque and colleagues reported in 2011 the use of eculizumab (an antibody directed against complement protein C5 used to treat atypical HUS, a disease that may arise due to complement dysregulation) in three children with HUS due to STEC infection (100). Although the sick children exhibited improvements in platelet counts and reductions in lactate dehydrogenase, plasma exchange and hemodialysis were used concurrently. In addition, no children in the small study received just antibody infusion alone. Eculizumab was also used to treat many patients with HUS during the O104:H4 outbreak in Germany in 2011. Although there was no evidence of efficacy for eculizumab in treating HUS from the outbreak in Germany, the cohorts are difficult to compare because the patients received other concurrent treatments, such as plasma exchange, anti-biotic therapy, immunoglobulin G immunoadsorption, and dialysis (14, 101–105). In addition, many of the HUS patients given eculizumab were quite ill and had neurological complications. Therefore, a randomized controlled trial is necessary to answer the question about the efficacy of eculizumab for the treatment of Stx-associated HUS.

**CONCLUSION**

Since the first outbreak of STEC infection in the United States in 1982, much research has focused on ways to neutralize the action of the Stxs. At this time, only SYNSORB Pk, urtoxazumab, and the Shiga monoclonal antibodies coStx1 and coStx2 have been tested in phase I and II trials intended to treat or prevent HUS. Efforts to
prevent STEC infection, such as elimination from the food supply and proper food handling, are important as well as we try to stop the development of the potentially deadly HUS. Lastly, consideration should be given to the development of a vaccine against the Stxs, minimally for those in research or clinical labs who are exposed to STEC.

ACKNOWLEDGMENT
We declare no conflicts of interest with regard to the manuscript.

REFERENCES


