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REVIEW

Recent Progress of Shiga Toxin Neutralizer for Treatment of Infections by Shiga Toxin-Producing *Escherichia coli*

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Abstract Infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC), including O157:H7, causes bloody diarrhea and hemorrhagic colitis in humans, occasionally resulting in fatal systemic complications, such as neurological damage and hemolytic-uremic syndrome. Because Stx is a major virulence factor of the infectious disease, a series of Shiga toxin neutralizers with various structural characteristics has been developed as promising therapeutic agents. Most of these agents function to bind to the toxin directly and inhibit the binding to its receptor present on the target cells. Other neutralizers do not inhibit receptor binding but induce aberrant intracellular transport of the toxin, resulting in effective detoxification. Such a novel type of Stx neutralizer provides a new therapeutic strategy against STEC infections. Here, recent progress of the development of Stx neutralizers is reviewed.

Keywords Shiga toxin · Neutralizer · Shiga toxin-producing *E. coli* · Globotriaosyl ceramide · Peptide-library · Clustering effect

Abbreviations

Stx	Shiga toxin
STEC	Shiga toxin-producing *Escherichia coli*
E. coli	*Escherichia coli*
HUS	Hemolytic-uremic syndrome
Gb3	Globotriaosyl ceramide
Gb4	Globotetraosyl ceramide
HuSAP	Human serum amyloid P component
ER	Endoplasmic reticulum

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Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) causes bloody diarrhea and hemorrhagic colitis in humans. These gastrointestinal diseases are often complicated by potentially fatal systemic sequelae such as neurological damage and hemolytic-uremic syndrome (HUS), the leading cause of acute renal failure in children (Karmali et al. 1983; O'Brien and Holmes 1987; Paton and Paton 1998; Riley et al. 1983). Stx produced in the gut lumen is closely related to gastrointestinal diseases. On the other hand, a small portion of Stx traverses the epithelium and then translocates to the circulation, where it causes vascular damage in specific target tissues such as the brain and kidneys, resulting in systemic complications. Thus, developing a neutralizer that specifically binds to and inhibits Stx in the gut and/or in the circulation could be a promising therapeutic approach.

Stx consists of a catalytic A subunit that has 28S rRNA *N*-glycosidase activity and inhibits eukaryotic protein synthesis and a pentameric B-subunit that is responsible for binding to the functional cell surface receptor, globotriaosyl ceramide (Gb3; Galα(1–4)-Galβ(1–4)-Glcβ1-ceramide) (Karmali et al. 1985; Melton-Celsa and O'Brien 1998; Paton and Paton 1998). Highly selective and potent binding of Stx to Gb3 is mainly attributed to the multivalent interaction between the B-subunit pentamer and the trisaccharide moiety of Gb3. This is sometimes referred to as the "clustering effect." On the basis of these facts, Stx neutralizers, in which the trisaccharide moiety of Gb3 or a

novel type of the B-subunit-binding unit is combined with various core structures in multiple configurations, have been developed.

Molecular Basis for Stx to Interact with Its Cell Surface Receptor

Stx is classified into two subgroups, Stx1 and Stx2, which have 56 and 58% amino acid and DNA sequence identity, respectively (Calderwood et al. 1987; DeGrandis et al. 1987; Jackson et al. 1987a; Jackson et al. 1987b; Kozlov et al. 1988; Strockbine et al. 1988; Willshaw et al. 1987; Yutsudo et al. 1987). A variant of Stx1, named Stx1c (Zhang et al. 2002), and several variants of Stx2, named Stx2c (Schmitt et al. 1991), Stx2d (Piérard et al. 1998), Stx2d-activatable (Kokai-Kun et al. 2000), Stx2e (Gannon et al. 1988), and Stx2f (Schmidt et al. 2000), which differ in their biological activities and association with disease, have been reported (Gyles 2007). All of these variants also recognize Gb3 as a receptor, except Stx2e, which prefers globotetraosyl ceramide (Gb4; GalNAcβ(1–3)-Galα(1–4)-Galβ(1–4)-Glcβ1-ceramide) to Gb3 (DeGrandis et al. 1989; Samuel et al. 1990). Epidemiological and experimental studies indicate that Stx2 is more closely related to the severity of STEC infections, such as the onset of neurological damage and HUS, than Stx1 (Donohue-Rolfe et al. 2000; Ostroff et al. 1989; Siegler et al. 2003; Tesh et al. 1993), suggesting the necessity to develop effective neutralizers, particularly against Stx2. The crystal structure of Stx1 revealed the presence of three distinctive binding sites (i.e., sites 1, 2, and 3) on each B-subunit monomer for the trisaccharide moiety of Gb3 (Ling et al. 1998). Accordingly, there are 15 potential trisaccharide-binding sites per pentamer, contributing to the high-affinity interaction between the B-subunit and Gb3. On the other hand, analysis of the crystal structure of Stx2 demonstrated that the conformation of Stx2 at site 2 distinctively differs from that of Stx1 compared with the other trisaccharide-binding sites (Fraser et al. 2004). In particular, the presence of Ser54 in site 2 of the Stx2 B-subunit is likely to hamper the accession of the penultimate galactose of the trisaccharide, suggesting that site 2 of Stx2 may only slightly contribute to trisaccharide binding (Fraser et al. 2004).

Kinetic analysis of the binding of Gb3 present in synthetic phospholipid vesicles to the Stx1 B-subunit or its mutants with amino acid substitutions in each trisaccharide-binding site demonstrated that all of the trisaccharide-binding sites of the Stx1 B-subunit are involved in the binding of Gb3 under physiological conditions (Nishikawa et al. 2005; Soltyk et al. 2002). In contrast, using the same assay system, it was shown that trisaccharide-binding sites 1 and 3, but not site 2, of the Stx2 B-subunit play significant roles in the physiological binding to Gb3 (Nishikawa et al. 2005). The lower contribution of site 2 present in Stx2 may be explained by the structural difference at this site between Stx1 and Stx2 as described above. It was also shown that the interaction with Gb3 is clearly distinguishable between Stx1 and Styx2 by analyzing their binding to a series of deoxy Gb3 analogs (Nyholm et al. 1996), further supporting that the availability of the trisaccharide-binding sites are different between these Stxs. Thus, to develop effective neutralizers, it is important to target the individual trisaccharide binding site(s) that play(s) pivotal roles in the receptor binding of each Stx to the practical target cells or organs in vivo.

Stx-Neutralizers with Clustered Trisaccharides Functioning in the Circulation

Because the multivalent interaction between the Stx B-subunit and the trisaccharide moiety of Gb3 is essential for high-affinity binding, a series of compounds with clustered trisaccharides, which can strongly bind to Stx and inhibit its cytotoxic activity, have been developed. Some of these neutralizers were designed to function in the circulation after being directly injected intravenously or subcutaneously.

A compound that consists of glucose as a core structure and pairs of trisaccharides at the tips of each spacer arm extended from the glucose, named STARFISH, was developed to permit pentavalent interactions with Stx (Kitov et al. 2000). STARFISH bound to both Stx1 and Stx2 with similarly high affinity and inhibited their cytotoxicity in Vero cells (Kitov et al. 2000). Crystallography of the STARFISH/Stx1 complex indicated that site 2 is exclusively involved in the binding. STARFISH protected mice from the lethality of Stx1, but not that of Stx2, when injected subcutaneously with the toxin (Mulvey et al. 2003). A modified version of STARFISH, called Daisy, in which the nature and length of the linkage between the terminal trisaccharide and the backbone were changed to increase the flexibility of the tether, successively protected mice from the lethality of both Stx1 and Stx2 when injected subcutaneously (Mulvey et al. 2003). Furthermore, Daisy showed protective effects in mice against oral challenge with STEC expressing two variant forms of Stx2, Stx2d1 and Stx2d2 (Mulvey et al. 2003).

A series of carbosilane dendrimers, in which trisaccharides are variously oriented at their termini in a conformation referred to as SUPER TWIG, has been synthesized (Matsuoka et al. 1999). SUPER TWIGs are unique in that the number of the terminal trisaccharides can be easily changed by regulating the number of silicon atoms present in the core structure. We identified a SUPER TWIG

carrying six trisaccharides, named SUPER TWIG(1)6, as the first Stx neutralizer that completely suppressed the lethal effect of Stx2 in mice when intravenously injected with the toxin (Fig. 1) (Nishikawa et al. 2002). Moreover, SUPER TWIG(1)6 protected mice from challenge with a fatal dose of STEC that produces Stx1 and Stx2, even when administered after the establishment of infection. SUPER TWIG(1)6 neutralizes Stx in vivo by a unique mechanism in which the accumulation and immediate degradation of Stx by phagocytic macrophages present in the reticuloendothelial system are induced after complex formation between SUPER TWIG(1)6 and Stx (Fig. 2) (Nishikawa et al. 2002).

The essential structures required for SUPER TWIG to function in the circulation were elucidated using a series of SUPER TWIGs with different structures (Fig. 1). A SUPER TWIG construct with 18 trisaccharides, named SUPER TWIG(2)18, was identified as another potent Stx neutralizer. SUPER TWIG(2)18 inhibited the cytotoxicity of Stx1 and Stx2 in Vero cells and completely suppressed the lethal effect of Stx2 in mice after intravenous injection (Nishikawa et al. 2005). Furthermore, SUPER TWIG(2)18 induced effective uptake of Stx2 by macrophages via complex formation. SUPER TWIG (1)12 and (2)36, which have 12 and 36 trisaccharides in their ball-shaped structures, respectively, bound Stx2 with even higher affinity than that of SUPER TWIG (1)6 and inhibited the cytotoxicity of the toxin in Vero cells. These SUPER TWIGs, however, did not inhibit the lethality of intravenously administered Stx2 in mice or induce the uptake of the toxin by macrophages (Nishikawa et al. 2005). All of these results indicate that the common structure between SUPER TWIG(1)6 and SUPER TWIG(2)18, where two clusters of trisaccharides are connected via a linkage with a hydrophobic core structure, is required for formation of a complex with Stx that enables efficient uptake and degradation of Stx by macrophages.

Using a series of Stx B-subunit mutants, it was found that among the three trisaccharide binding sites, site 3 plays a pivotal role in the high-affinity binding of SUPER TWIG(1)6 and SUPER TWIG(2)18 to either Stx1 or Stx2 B-subunits. Of note, regarding Stx2, site 3 has been determined to be an exclusive binding site of both SUPER TWIG(1)6 and SUPER TWIG(2)18, as a double mutant of the Stx2 B-subunit that has amino acid substitutions both at site 1 and site 2 can still bind these SUPER TWIGs with high affinity (Nishikawa et al. 2005).

A supramolecular templating strategy has been used to design a high-affinity neutralizer of Stx. In this strategy, a polymeric heterobifunctional molecule, named (*S*)-Poly-BAIT, which recognizes both Stx1 and human serum amyloid P component (HuSAP) through interactions with its trisaccharide moiety and a cyclic pyruvate ketal moiety, respectively, was developed (Kitov et al. 2008). HuSAP, which is a circulating plasma protein and a member of the highly conserved pentraxin family, was used as a template protein. In the presence of HuSAP, (*S*)-PolyBAIT binds to Stx1 with high affinity through the formation of a ternary sandwich type complex because the HuSAP organizes the configuration of the trisaccharide moiety to efficiently

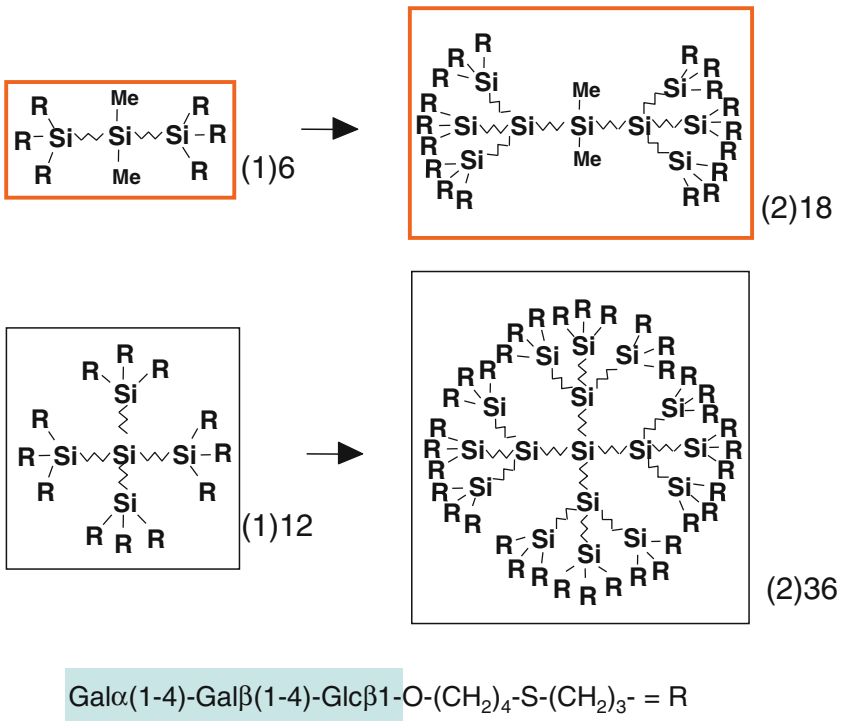

Fig. 1 Structure of SUPER TWIGs. The numbers in the parentheses indicate the generation number of the SUPER TWIGs. The first and the second generations of the SUPER TWIGs have three and five linearly arranged silicon atoms, respectively, in their core structures. The *numbers* at the right of the parentheses indicate the number of trisaccharides present in the SUPER TWIGs. SUPER TWIGs (1)6 and (2)18 have a dumbbell-shaped structure, and SUPER TWIGs (1)12 and (2)36 have a ball-shaped structure

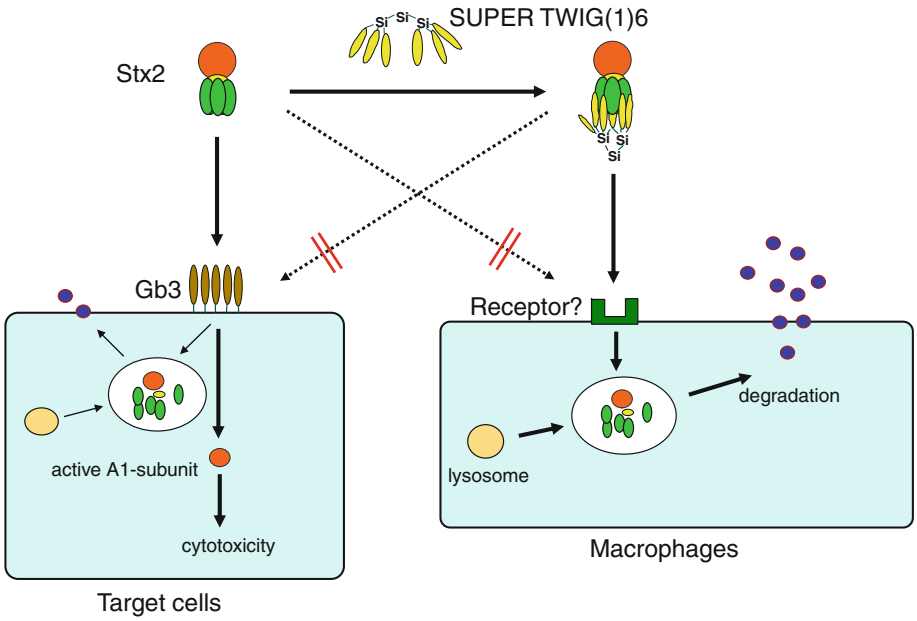

Fig. 2 Mechanism of the Stx2-neutralizing action of SUPER TWIG(1)6 in vivo. SUPER TWIG(1)6 forms a complex with Stx2 and inhibits the cytotoxicity of the toxin by blocking its uptake into the target cells. This complex, however, is effectively incorporated by macrophages present in the reticuloendothelial system, resulting in degradation of the toxin. In the absence of SUPER TWIG(1)6, Stx2 is not incorporated by macrophages

interact with Stx. Using transgenic mice that constitutively express HuSAP, it was demonstrated that (*S*)-PolyBAIT protected mice from the lethality of Stx1 when it was administered intravenously with the toxin (Kitov et al. 2008). Stx2, which is the most clinically significant subtype, cannot be evaluated in this animal model because HuSAP was shown to form a complex with Stx2 by itself (Kimura et al. 2001).

Orally Applicable Stx-Neutralizers with Clustered Trisaccharides

Oral administration of a receptor blocker has the theoretic advantage of neutralizing the toxin before the toxin translocates systemically. An initial study was undertaken using a compound in which trisaccharides were covalently coupled to Chromosorb P (inert silica particles) through an alky spacer named Synsorb-Pk (Armstrong et al. 1991). Although Synsorb-Pk bound to and inhibited the cytotoxicity of Stx1 and that of Stx2 to a lesser extent, oral administration of Synsorb-Pk failed to diminish the severity of diarrhea-associated HUS in a randomized, double-blind, placebo-controlled clinical trial in children (Trachtman et al. 2003). A probable explanation is that Synsorb-Pk has a lower density of the trisaccharide on its surface and steric hindrance to bind Stx released in close proximity to the epithelial surface from the intimately attached STEC.

Based on a probiotic approach, a harmless bacterium expressing closely packed trisaccharides on its surface was developed as a therapeutic receptor mimicking structure (Paton et al. 2006). A genetically engineered non-pathogenic *E. coli* strain that expressed a chimeric lipopolysaccharide terminating in Galα(1–4)-Galβ(1–4)-Glc, named CWG308:pJCP-Gb3, was generated by introducing two *Neisseria* galactosyl transferase genes (*lgtC* and *lgtE*) (Paton et al. 2000). Direct and specific binding of Stx to the bacterial surface was observed. Oral administration of the recombinant bacterium completely protected streptomycin-treated mice against an otherwise 100% fatal dose of B2F1, the most mouse-virulent strain used in this model (Paton et al. 2000). This protective effect in mice was confirmed using formaldehyde-killed CWG308:pJCP-Gb3 bactereum, although it was necessary to increase the frequency of administration to maintain 100% protection (Paton et al. 2001b). This strategy was proven effective against another Stx family member, Stx2e, which is produced by a subgroup of STEC strains that causes edema in piglets. As Stx2e recognizes Gb4 (DeGrandis et al. 1989), the CWG308:pJCP-Gb3 bacterium was modified to express globotetraose by introducing an additional *N*-acetylgalactosamine transferase gene. The obtained bacteria had a reduced capacity to neutralize Stx1 and Stx2c in vitro but effectively neutralized the cytotoxicity of Stx2e (Paton et al. 2001a).

We developed a series of linear polymers of acrylamide, each with a different density of the trisaccharide through a spacer from the core structure, named Gb3 polymers (Watanabe et al. 2004). The Gb3 polymers with highly clustered trisaccharides specifically bound to both Stx1 and Stx2 with high affinity and inhibited their cytotoxicity (Watanabe et al. 2004). Oral administration of the Gb3 polymers protected mice from challenge with a fatal dose

of STEC, with marked reduction of the serum level of Stx and fatal brain damages, suggesting that the Gb3 polymers entrap Stx in the gut and prevent its entrance into the circulation (Watanabe et al. 2004).

Using a Gb3 polymer with a short spacer tethering the trisaccharide to the core, it was found that shortening the spacer length markedly reduced the binding affinity for Stx2, but not for Stx1 (Watanabe et al. 2006). Mutational analysis of Stx revealed that the essential trisaccharide-binding sites for the Gb3 polymer were completely different between Stx1 and Stx2 in that site 2 and site 3 were indispensable for the high-affinity binding to Stx1 and Stx2, respectively, irrespective of the spacer length of the polymer (Watanabe et al. 2006). Thus, for the Gb3 polymer, both the occupation of site 3 by the trisaccharide moiety of the polymer and the hydrophobic interaction in this region through the alkyl spacer attached to the trisaccharide are required for the substantial inhibition of Stx2.

Peptide-Based Stx-Neutralizers

As mentioned earlier, some of the synthetic Stx neutralizers with clustered trisaccharides have been proven effective in STEC infection experiments in mice. The clinical application of these neutralizers, however, has been substantially hampered by the synthetic complexity of its trisaccharide moiety. Furthermore, the trisaccharide itself, if not clustered, has a very low affinity for Stx ($K_d = 1 \times 10^{-3}$ M), even though it is a natural Stx-binding unit. To develop a novel Stx-binding unit that overcomes these problems, other widely used techniques, including screening of chemical compound libraries and phage display libraries, are possible approaches. These trials, however, have not been successful because they are not theoretically based on the "clustering effect."

Previously, an affinity-based peptide library technique was used to develop a specific inhibitor of ZAP-70 Tyr-kinase, a protein kinase involved in T cell activation (Nishikawa et al. 2000). The identified peptide-based inhibitor directly bound to the catalytic site of ZAP-70 with high affinity and high selectivity and successfully blocked downstream signaling of ZAP-70 when introduced into intact T cells (Nishikawa et al. 2000). We improved this technique to exploit the "clustering effect" by synthesizing a tetravalent peptide library. The library has a polylysine core bifurcating at both ends with four randomized peptides (Fig. 3a). The core structure, including the length of the spacers, was optimized for high-affinity binding to the Stx B-subunit pentamer, based on the following structural requirement that has been established using a series of SUPER TWIGs: (1) a dumbbell-shaped structure in which two clusters of trisaccharides are connected to a hydrophobic core structure with a length of at least 11 Å is required; and (2) when the dumbbell shape is present, at least four trisaccharides are sufficient for high-affinity binding to the Stx B-subunit and for Stx-neutralizing activities in vitro (Nishikawa et al. 2005).

The tetravalent peptide library was screened to locate compounds with the ability to bind to the Stx2 B-subunit but not mutant B-subunit that has a single amino acid substitution in trisaccharide-binding site 3 to identify site 3-specific binding motifs (Fig. 3a). This site was selected as a target site because all SUPER TWIGs that bind to the B-subunit with high affinity bind exclusively to site 3 (Nishikawa et al. 2005). After affinity-driven two-round screening, we have identified four peptide motifs that are superior to trisaccharide as an Stx-binding unit (Fig. 3b) (Nishikawa et al. 2006). Tetravalent forms of these peptides bind to trisaccharide-binding site 3 with high affinity and effectively inhibit Stx2 cytotoxicity. PPP-tet, which is the tetravalent form of one of the identified motifs, Pro-Pro-Pro-Arg-Arg-Arg-Arg, almost completely protected mice from a fatal dose of *E. coli* O157:H7, even when orally administered after an established infection (Nishikawa et al. 2006). Moreover, the addition of acetyl groups to all the amino termini of PPP-tet (Ac-PPP-tet) confers proteolytic resistance to this compound and markedly enhances its protective activity against STEC infection (Nishikawa et al. 2006). Treatment with Ac-PPP-tet beginning on day 3 of infection, when the serum concentration of the inflammatory cytokine tumor necrosis factor α reaches peak levels, still protected mice from lethality. The lowest effective amount of Ac-PPP-tet used can be extrapolated to be 0.53 g/70 kg of body weight for human therapy, indicating that this compound holds promise as a therapeutic agent for STEC infections.

Retrograde Transport Process as a Practical Target for a Novel Stx Neutralizer

After binding to Gb3, Stx is first transported to the Golgi apparatus in a retrograde manner, and then transported to the endoplasmic reticulum (ER) where its catalytic A-subunit is released into the cytosol to inhibit protein synthesis (Sandvig et al. 1992; Sandvig and van Deurs 2000). Although this retrograde transport is essential for Stx to exert its cytotoxic activity, an Stx neutralizer that specifically inhibits this transport process had not been developed.

Interestingly, PPP-tet was found to inhibit the cytotoxicity of Stx2 by inducing its aberrant intracellular transport, not by preventing its binding to the cell surface receptor. PPP-tet formed a complex with Stx2, which was effectively incorporated into the cells and transferred to the Golgi but

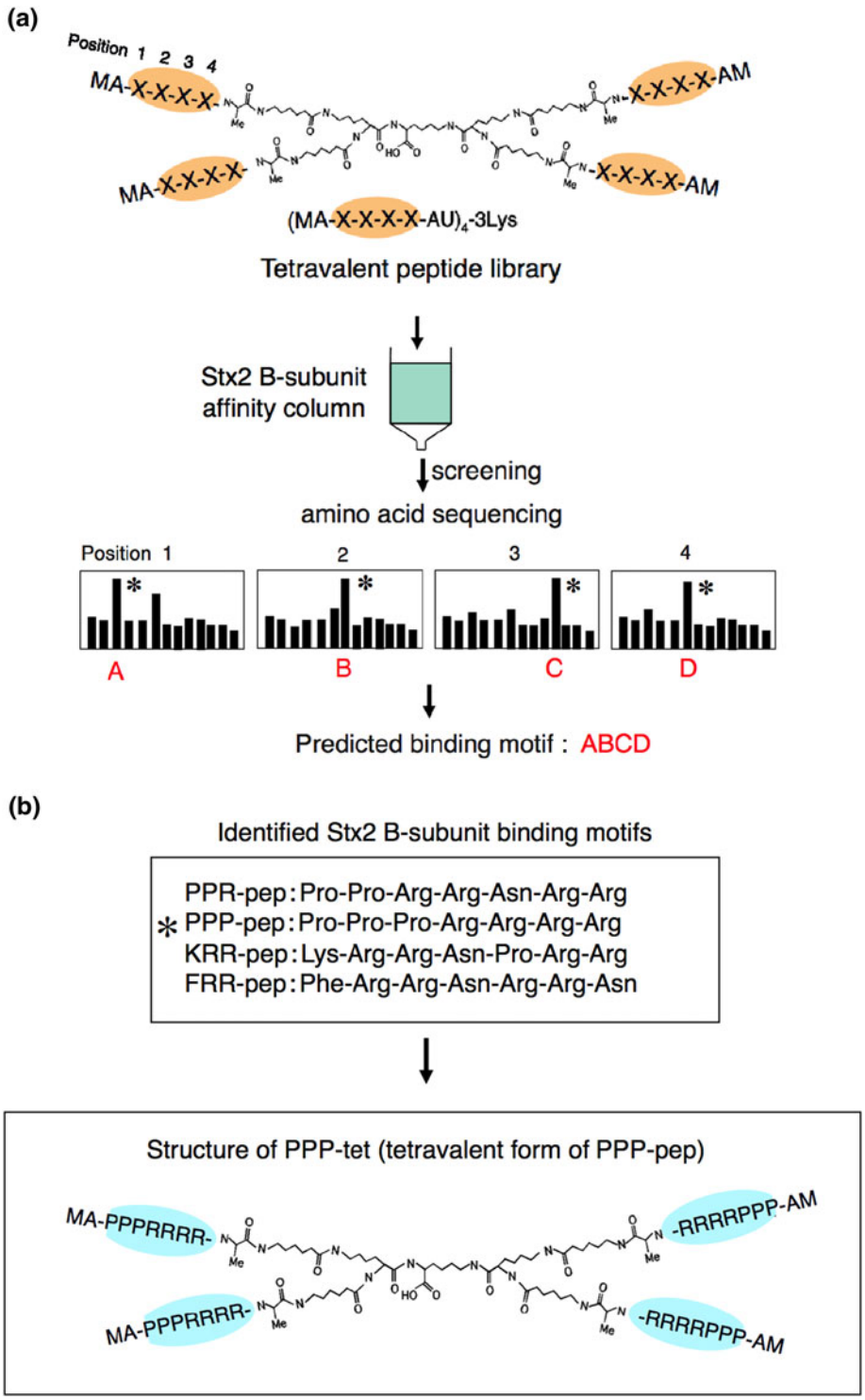

Fig. 3 Multivalent peptide-library screening. **a** Identification of Stx2 B-subunit binding motifs by using a tetravalent peptide library technique. To exert the "clustering effect," a tetravalent peptide-library with four randomized peptides was used for screening. Each randomized peptide of the library (first library) has the sequence Met-Ala-X-X-X-X-Ala-U (U; amino hexanoic acid as a spacer), where X indicates any amino acid except Cys. The library was screened for compounds that could bind to the Stx B-subunit, but not its mutant with amino acid substitution at trisaccharide-binding site 3. After amino acid sequencing of the screened library, preferred amino acids at each degenerated position were determined. Based on the results, tetravalent peptide libraries with fixed Arg and/or Asn were synthesized as the second library and used for the second round of selections to identify the site 3-specific peptide motifs. **b** The obtained peptide motifs, and the structure of PPP-tet, the tetravalent form of one of the identified motifs, PPP-peptide, are shown

not to the ER (Nishikawa et al. 2006). Instead, the complex was transferred from the Golgi to an acidic compartment, which resulted in the efficient degradation of Stx2. Furthermore, due to its cell-permeable nature, PPP-tet inhibited the cytotoxicity of Stx even when added up to 3 h after the incorporation of the toxin, through the same mechanism (Nishikawa et al. 2006). Such an inhibitory activity was not observed with other Stx neutralizers with clustered trisaccharides, such as SUPER TWIG (1)6, which function to inhibit the binding of Stx to target cells.

How does PPP-tet induce the aberrant cellular transport of Stx2? PPP-tet exclusively binds to site 3 of the B-subunit, leaving the residual sites 1 and 2 intact. Then, the complex binds to the target cells not only due to the

cell-permeable nature of PPP-tet but also due to the interaction between the residual trisaccharide-binding sites and Gb3 present on the cell surface. This unique complex formation is followed by its incorporation into the cells, resulting in the induction of aberrant intracellular transport of the toxin as described above (Fig. 4) (Nishikawa et al. 2006). These observations indicate that binding of Gb3 through site 1 and/or 2 of Stx2 is required for the efficient retrograde transport to the Golgi, whereas binding of Gb3 through site 3 plays an essential role in its transfer from the Golgi to the ER. A complex of PPP-tet with a double-mutant Stx2 B-subunit that has amino acid substitutions at sites 1 and 2 can be incorporated into cells but not transferred to the Golgi, further supporting this contention (Nishikawa et al. 2006). Thus, the partially cell-permeable nature of PPP-tet enabled analysis of the specific role of each trisaccharide-binding site in the retrograde transport of Stx. Such an analysis, however, cannot be practically achieved using various Stx mutants because any mutation of any trisaccharide-binding site results in marked reduction of Stx binding to target cells (Nishikawa et al. 2005; Soltyk et al. 2002). The precise molecular mechanism by which each trisaccharide-binding site exerts its specific function remains to be elucidated.

To understand how orally administered tetravalent peptides, such as Ac-PPP-tet, function in vivo, the effect of

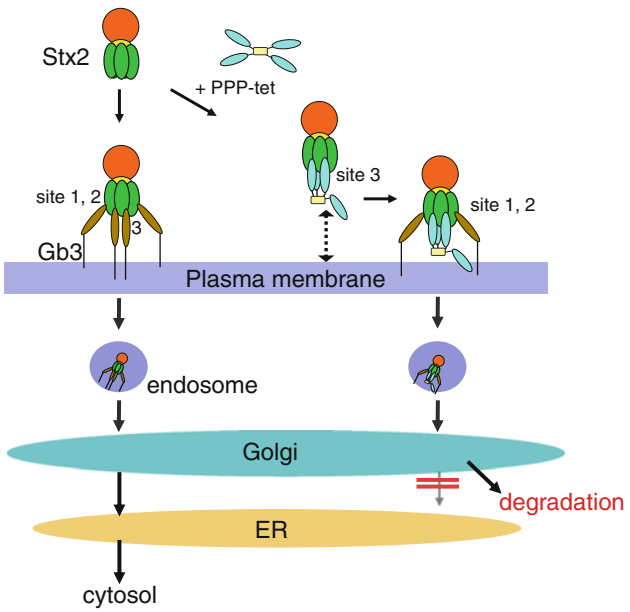

Fig. 4 Mechanism of action by which PPP-tet inhibits Stx2 in target cells. PPP-tet forms a complex with Stx2 through traisaccharide-binding site 3, and the complex associates with target cells due to the cell-permeable nature of PPP-tet. On the cell surface, Stx2 and Gb3 form a complex via an interaction at the residual site(s), site 1 and/or site 2, followed by the intracellular transport of the toxin to the Golgi and then to the acidic compartment, rather than to the ER, for its effective degradation

Ac-PPP-tet on fluid accumulation in the rabbit ileum caused by the direct injection of Stx2 was investigated. This system is highly valid for evaluating the toxicity of Stx2 produced in the intestine after infection. Ac-PPP-tet completely inhibited fluid accumulation in the ileum, where Ac-PPP-tet accumulated in the ileal epithelial cells only through the formation of a complex with Stx2 (Watanabe-Takahashi et al. 2010). This complex formation also blocked the intracellular transport of Stx2 from the Golgi to the ER in the epithelial cells. All of these observations indicate that orally administered Ac-PPP-tet forms a complex with Stx2 in the ileum, which is effectively incorporated into epithelial cells and induces aberrant transport of the toxin for its detoxification through the same mechanism described above. Thus, it is highly promising to regulate the intracellular transport of Stx in target cells for the practical treatment of STEC infections.

Concluding Remarks

Stx neutralizers with clustered trisaccharides that function in the circulation, i.e., Daisy, SUPER TWIG (1)6, and (*S*)-PolyBAIT, form a complex with Stx and then cause marked accumulation of the toxin in reticuloendothelial system organs such as the liver and spleen, resulting in its efficient detoxification (Kitov et al. 2008; Mulvey et al. 2003; Nishikawa et al. 2002). Thus, the capability of an Stx neutralizer to bind to Stx with high affinity is not sufficient for it to substantially function in the circulation. The significance of marked accumulation of the toxin in the reticuloendothelial system was clearly confirmed using SUPER TWIG (1)12 and (2)36, both of which bind to Stx2 with high affinity but do not inhibit the lethality of intravenously administered Stx2 in mice or induce the uptake of the toxin by macrophages. In many cases of STEC infection, Stx is translocated from the gut into the circulation by the time of the appearance of symptoms, such as bloody diarrhea and hemorrhagic colitis, and quickly distributes to the target tissues with very short serum half-life (less than 5 min) (Richardson et al. 1992). Given such characteristics of Stx, intravenously applicable Stx neutralizers that bind Stx with high affinity to block the prompt distribution of the toxin to target tissues and induce its effective degradation in the reticuloendothelial system are expected to be viable therapeutics.

Orally applicable Stx neutralizers, such as Gb3-polymers, CWG308:pJCP-Gb3, and Ac-PPP-tet, can theoretically neutralize the toxin in the gut before its systemic translocation. In addition, this type of Stx neutralizer can be easily and widely applicable not only to patients with gastrointestinal symptoms but also to suspected individuals at risk of STEC infection to prevent the onset of life-threatening

systemic complications. A merit of such prophylactic usage was further confirmed by the observation that the oral administration of these neutralizers can be delayed for up to 48–72 h after STEC challenge and still have significant protective effects, at least in the mouse model (Nishikawa et al. 2006; Paton et al. 2001b; Watanabe et al. 2004). In particular, Ac-PPP-tet, which has a peptide-based structure without trisaccharide moieties, holds promise as a practical therapeutic agent because this compound can be easily synthesized using N-α-FMOC-protected amino acid and standard BOP/HOB coupling chemistry.

Identification of the inhibitory mechanisms of PPP-tet and Ac-PPP-tet, which do not inhibit the Stx binding to its cell surface receptor but induce aberrant cellular transport and subsequent degradation of the toxin, will facilitate the development of a series of novel Stx neutralizers that regulate the intracellular transport of the toxin at various steps. Such neutralizers are expected to be developed using tetravalent peptide library techniques targeting the specific trisaccharide-binding sites of Stx1 and Stx2 because each binding site may play a different role in the intracellular transport process of Stx as describe above. Very recently, high-throughput chemical library screening identified two compounds that inhibit the cytotoxicity of Stx and ricin, a plant toxin that also has *N*-glucosidase activity, by blocking their intracellular retrograde transport at the early endosome-*trans*-Golgi network (Stechmann et al. 2010). One of the compounds protects mice from the lethality of nasal exposure to ricin, although this compound has to be administered prior to ricin exposure. All of these findings further confirm the effectiveness of regulating the retrograde transport process of Stx as a potential therapeutic target.

References

Armstrong GD, Fodor E, Vanmaele R (1991) Investigation of Shiga-like toxin binding to chemically synthesized oligosaccharide sequences. J Infect Dis 164:1160–1167

Calderwood SB, Auclair F, Donohue-Rolfe A et al (1987) Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. Proc Natl Acad Sci USA 84:4364–4368

DeGrandis S, Ginsberg J, Toone M et al (1987) Nucleotide sequence and promoter mapping of the *Escherichia coli* Shiga-like toxin operon of bacteriophage H-19B. J Bacteriol 169:4313–4319

DeGrandis S, Law H, Brunton J et al (1989) Globotetraosylceramide is recognized by the pig edema disease toxin. J Biol Chem 264:12520–12525

Donohue-Rolfe A, Kondova I, Oswald S et al (2000) *Escherichia coli* O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2. J Infect Dis 181:1825–1829

Fraser ME, Fujinaga M, Cherney MM et al (2004) Structure of Shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. J Biol Chem 279:27511–27517

Gannon VP, Gyles CL, Friendship RW (1988) Characteristics of verotoxigenic *Escherichia coli* from pigs. Can J Vet Res 52:331–337

Gyles CL (2007) Shiga toxin-producing *Escherichia coli*: an overview. J Anim Sci 85(13 suppl):E45–E62

Jackson MP, Neill RJ, O'Brien AD et al (1987a) Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol Lett 44:109–114

Jackson MP, Newland JW, Holmes RK et al (1987b) Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933 J from *Escherichia coli*. Microb Pathog 2:147–153

Karmali MA, Steele BT, Petric M et al (1983) Sporadic cases of hemolytic uremic syndrome associated with fecal cytotoxin and cytotoxin-producing *Escherichia coli*. Lancet 1:619–620

Karmali MA, Petric M, Lim C et al (1985) The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J Infect Dis 151:775–782

Kimura T, Tani S, Matsumoto Y et al (2001) Serum amyloid P component is the Shiga toxin 2-neutralizing factor in human blood. J Biol Chem 276:41576–41579

Kitov PI, Sadowska JM, Mulvey G et al (2000) Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. Nature 403:669–672

Kitov PI, Mulvey GL, Griener TP et al (2008) In vivo supramolecular templating enhances the activity of multivalent ligands: a potential therapeutic against the *Escherichia coli* O157 AB5 toxins. Proc Natl Acad Sci USA 105:16837–16842

Kokai-Kun JF, Melton-Celsa AR, O'Brien AD (2000) Elastase in intestinal mucus enhances the cytotoxicity of Shiga toxin type 2d. J Biol Chem 275:3713–3721

Kozlov YV, Kabishev AA, Lukyanov EV et al (1988) The primary structure of the operons coding for Shigella dysenteriae toxin and temperature phage H30 Shiga-like toxin. Gene 67:213–221

Ling H, Boodhoo A, Hazes B et al (1998) Structure of the shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb3. Biochemistry 37:1777–1788

Matsuoka K, Terabatake M, Esumi Y et al (1999) Synthetic assembly of trisaccharide moieties of globotriaosyl ceramide using carbosilane dendrimers as cores. A new type of functional glyco-material. Tetrahedron Lett 40:7839–7842

Melton-Celsa AR, O'Brien AD (1998) Structure, biology, and relative toxicity of Shiga toxin family members for cells and animals. In: Kaper JB, O'Brien AD (eds) *Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Strains. Am Soc Microbiol, Washington, pp 121–128

Mulvey GL, Marcato P, Kitov PI et al (2003) Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. J Infect Dis 187:640–649

Nishikawa K, Sawasdikosol S, Fruman DA et al (2000) A peptide library approach identifies a specific inhibitor for the ZAP-70 protein tyrosine kinase. Mol Cell 6:969–974

Nishikawa K, Matsuoka K, Kita E et al (2002) A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin-producing *Escherichia coli* O157:H7. Proc Natl Acad Sci USA 99:7669–7674

Nishikawa K, Matsuoka K, Watanabe M et al (2005) Identification of the optimal structure for a Shiga toxin neutralizer with oriented carbohydrates to function in the circulation. J Infect Dis 191:2097–2105

Nishikawa K, Watanabe M, Kita E et al (2006) A multivalent peptide-library approach identifies a novel Shiga toxin-inhibitor that induces aberrant cellular transport of the toxin. FASEB J 20:2597–2599

Nyholm PG, Magnusson G, Zheng Z et al (1996) Two distinct binding sites for globotriaosyl ceramide on verotoxins: identification by

molecular modelling and confirmation using deoxy analogues and a new glycolipid receptor for all verotoxins. Chem Biol 3:263–275
O'Brien AD, Holmes RK (1987) Shiga and Shiga-like toxins. Microbiol Rev 51:206–220
Ostroff SM, Tarr PI, Neill MA et al (1989) Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J Infect Dis 160:994–998
Paton JC, Paton AW (1998) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 11:450–479
Paton AW, Morona R, Paton JC (2000) A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. Nat Med 6:265–270
Paton AW, Morona R, Paton JC (2001a) Neutralization of Shiga toxins Stx1, Stx2c, and Stx2e by recombinant bacteria expressing mimics of globotriose and globotetraose. Infect Immun 69:1967–1970
Paton JC, Rogers TJ, Morona R et al (2001b) Oral administration of formaldehyde-killed recombinant bacteria expressing a mimic of the Shiga toxin receptor protects mice from fatal challenge with Shiga-toxigenic *Escherichia coli*. Infect Immun 69:1389–1393
Paton AW, Morona R, Paton JC (2006) Designer probiotics for prevention of enteric infections. Nat Rev Microbiol 4:193–200
Piérard D, Muyldermans G, Moriau L et al (1998) Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. J Clin Microbiol 36:3317–3322
Richardson SE, Rotman TA, Jay V et al (1992) Experimental verocytotoxemia in rabbits. Infect Immun 60:4154–4167
Riley LW, Remis RS, Helgerson SD et al (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 308:681–685
Samuel JE, Perera LP, Ward S et al (1990) Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. Infect Immun 58:611–618
Sandvig K, van Deurs B (2000) Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. EMBO J 19:5943–5950
Sandvig K, Garred O, Prydz K et al (1992) Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature 358:510–512
Schmidt H, Scheef J, Morabito S et al (2000) A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl Environ Microbiol 66:1205–1208
Schmitt CK, McKee ML, O'Brien AD (1991) Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511. Infect Immun 59:1065–1073
Siegler RL, Obrig TG, Pysher TJ et al (2003) Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. Pediatr Nephrol 18:92–96
Soltyk AM, MacKenzie CR, Wolski VM et al (2002) A mutational analysis of the globotriaosylceramide-binding sites of verotoxin VT1. J Biol Chem 277:5351–5359
Stechmann B, Bai SK, Gobbo E et al (2010) Inhibition of retrograde transport protects mice from lethal ricin challenge. Cell 141:231–242
Strockbine NA, Jackson MP, Sung LM et al (1988) Cloning and sequencing of the genes for Shiga toxin from Shigella dysenteriae type 1. J Bacteriol 170:695–700
Tesh VL, Burris JA, Owens JW et al (1993) Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect Immun 61:3392–3402
Trachtman H, Cnaan A, Christen E et al (2003) Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: a randomized controlled trial. JAMA 290:1337–1344
Watanabe M, Matsuoka K, Kita E et al (2004) Oral therapeutic agents with highly clustered globotriose for treatment of Shiga toxigenic *Escherichia coli* infections. J Infect Dis 189:360–368
Watanabe M, Igai K, Matsuoka K et al (2006) Structural analysis of the interaction between Shiga toxin B-subunits and linear polymers bearing clustered globotriose residues. Infect Immun 74:1984–1988
Watanabe-Takahashi M, Sato T, Dohi T et al (2010) An orally applicable Shiga toxin neutralizer functions in the intestine to inhibit the intracellular transport of the toxin. Infect Immun 78:177–183
Willshaw GA, Smith HR, Scotland SM et al (1987) Heterogeneity of *Escherichia coli* phages encoding Vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J Gen Microbiol 133:1309–1317
Yutsudo T, Kurazono H, Sasakawa C et al (1987) Cloning of a Vero toxin (VT2) gene from a VT2-converting phage isolated from *Escherichia coli* 0157:H7. FEMS Microbiol Lett 48:273–276
Zhang W, Bielaszewska M, Kuczius T et al (2002) Identification, characterization, and distribution of a Shiga toxin 1 gene variant (stx(1c)) in *Escherichia coli* strains isolated from humans. J Clin Microbiol 40:1441–1446