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Review

Cure and Curse: *E. coli* Heat-Stable Enterotoxin and Its Receptor Guanylyl Cyclase C

Philipp R. Weiglmeier, Paul Rösch and Hanna Berkner *

Lehrstuhl für Biopolymere und Forschungszentrum für Bio-Makromoleküle, Universität Bayreuth, Universitätsstraße 30, 95447 Bayreuth, Germany;
E-Mails: philipp.weiglmeier@uni-bayreuth.de (P.R.W.); roesch@unibt.de (P.R.)

* Author to whom correspondence should be addressed; E-Mail: hanna.berkner@uni-bayreuth.de.

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Abstract: Enterotoxigenic *Escherichia coli* (ETEC) associated diarrhea is responsible for roughly half a million deaths per year, the majority taking place in developing countries. The main agent responsible for these diseases is the bacterial heat-stable enterotoxin STa. STa is secreted by ETEC and after secretion binds to the intestinal receptor guanylyl cyclase C (GC-C), thus triggering a signaling cascade that eventually leads to the release of electrolytes and water in the intestine. Additionally, GC-C is a specific marker for colorectal carcinoma and STa is suggested to have an inhibitory effect on intestinal carcinogenesis. To understand the conformational events involved in ligand binding to GC-C and to devise therapeutic strategies to treat both diarrheal diseases and colorectal cancer, it is paramount to obtain structural information on the receptor ligand system. Here we summarize the currently available structural data and report on physiological consequences of STa binding to GC-C in intestinal epithelia and colorectal carcinoma cells.

Keywords: heat-stable enterotoxin; guanylyl cyclase C; secretory diarrhea; colorectal cancer

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1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause for acute secretory diarrhea in developing countries with insufficient sanitation and no adequate supply of clean water. These pathogens account for around a half million deaths per year, mostly of children in developing countries [1], and are the cause of traveller's diarrhea. ETEC, a very diverse group of pathogenic

E. coli, colonize the small intestine and produce the toxic agents heat-labile (LT) and heat-stable (ST) enterotoxin. Apparently, the acquisition of enterotoxin genes is sufficient for the expression of enterotoxigenicity [2]. Clinically, ETEC associated diarrhea is virtually indistinguishable from cholera [3]. The *E. coli* heat-labile toxin LT is a hexameric protein consisting of a single A subunit and a homopentameric B subunit. The two domain A subunit represents the actual toxin, which activates the guanine nucleotide protein Gsα by ADP-ribosylation and ultimately leads to stimulated secretion by a cAMP-dependent mechanism involving the cystic fibrosis transmembrane conductance regulator (CFTR). LT is structurally and functionally very similar to cholera toxin (CT)[4]. Heat-stable enterotoxins are small peptides that are secreted by enterotoxigenic bacteria. ST peptides are active even after 60 min of heating at 95 °C [5]. Two classes of STs that differ in structure and function can be distinguished: the methanol soluble protease resistant and guanylyl cyclase C (GC-C) binding STa and the methanol insoluble and protease sensitive STb. STb is a 48 amino acid peptide associated with disease in cattle, but not in humans, and it does not bind to GC-C. STb was shown to increase intracellular levels of Ca^{2+} [6].

STas are 18 or 19 amino acid cysteine-rich peptides that activate intestinal guanylyl cyclase C and induce secretion by cGMP-dependent activation of CFTR. Genetically, the toxins are encoded on plasmids [7]. In some cases they were found to be encoded in transposons or in sequences that were once part of a transposon [8,9].

Peptides that are highly homologous to *E. coli* STa have been identified in other enteric bacteria such as *Klebsiella pneumoniae* [10], *Yersinia enterolitica* [11], *Citrobacter freundii* [12], cholera toxin positive *Vibrio cholerae* O1 [13] and nonagglutinable *Vibrios* [14–16].

Other notable enterotoxins produced by pathogenic *E. coli* include the enteroaggregative heat-stable toxin EAST1 [17,18] which is structurally similar to STa, and Shiga toxin Stx produced by shiga-toxigenic *E. coli* (STEC)[19].

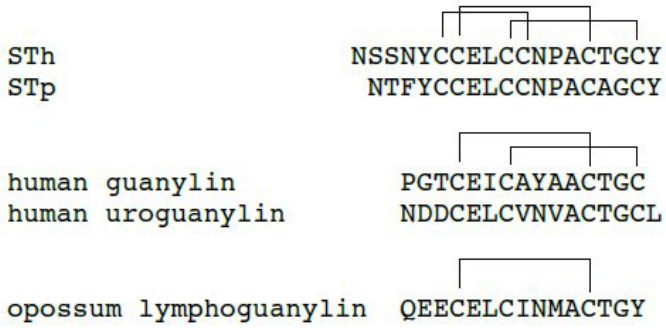
2. Structure of ST Peptides

The two most common STas produced by *E. coli* are STh and STp. These toxins consist of 18 (STp) or 19 (STh) amino acids including six cysteines that form three intramolecular disulfide linkages. Both peptides share the carboxy-terminal 14 residues which are sufficient for enterotoxicity and referred to as the toxic domain [20]. This toxic domain shows significant homology to the sequence of the mammalian endogenous peptides guanylin [21], uroguanylin [22] and lymphoguanylin [23]. In contrast, the amino-terminal four or five residues are neither homologous in the two ST species nor required for biological activity.

The mode of disulfide bond formation is identical in both STh and STp (Figure 1): In the STh numbering system, the bonds are Cys6–Cys11, Cys7–Cys15 and Cys10–Cys18 [24]. In order to determine the relative importance of each disulfide bond, STh analogs were synthesized that lack one or two disulfide linkages by pairwise replacement of cysteines with alanines [25]. Comparison of the ability of these analogs to inhibit the binding of radiolabeled [^{125}I-Y4]-STh(6–18) to intestinal cells revealed that the Cys7–Cys15 bond is crucial for biological activity. Replacement of the Cys6–Cys11 and Cys10–Cys18 bond results in peptides that bind 4200 and 130 times less strongly to their receptor, respectively, as measured by their IC_{50} values. The Cys7–Cys15 disulfide linkage alone, however, is not sufficient for binding [25,26].

The homologous mammalian peptides guanylin and uroguanylin both contain four cysteine residues that form two disulfide bonds with 1–3/2–4 connectivity (Figure 1), lacking one of the bonds present in *E. coli* STa. The two cysteine bonds give rise to topological isomerism in both peptides [27,28]. Only one of the interconvertible topoisomers of each peptide, the so-called A-form, is biologically active and binds to GC-C. The rate of interconversion differs in guanylin and uroguanylin. Guanylin interconversion occurs with a half-life of seconds while the process is much slower for uroguanylin [29]. The interconversion was shown to be controlled by sterical hindrance from the carboxy-terminal residues as well as by the side-chains in the central part of the peptides that modulates the flexibility of the chains [30,31].

Figure 1. The primary structure of *E. coli* STh and STp, human hormones guanylin, uroguanylin and opossum lymphoguanylin. Disulfide linkages are indicated by lines above.



Both STh and STp are synthesized as 72 amino acid precursor proteins consisting of a pre signal peptide, a pro sequence, and the carboxy-terminal enterotoxin [32,33]. The polypeptide is synthesized as an intracellular pre-pro-STa. The 19 amino acid signal sequence is cleaved during translocation from the cytoplasm to the periplasm by a SecA-dependent export pathway [34]. The exact function of the pro sequence, which is non-essential for enterotoxin secretion, is unclear [32], although the highly conserved region from residue 29 to 38 in the pro sequence greatly increases the translocation of STa across the inner membrane [35]. If amino acid residues 29 to 31 (K-E-K) are substituted by hydrophobic residues or by all-basic residues (K-K-K), the efficiency of STa delivery to the periplasm is significantly reduced. Conversely, an amino acid substitution to an all-acidic motif (E-E-E) leads to increased translocation of STa across the inner membrane. Some studies indicated that the pro sequence is cleaved off inside the periplasm where the thiol-disulfide oxidoreductase DsbA is thought to catalyse the disulfide bond formation in STa [36,37]. The mature STa is then believed to be secreted across the outer membrane. Other results are in conflict with this view as deletion of the pro sequence or the STa peptide leads to detection of STa or pro peptide in the supernatant of cells, clearly indicating that the pro region can cross the outer membrane [38]. Other studies showed that various fusion proteins consisting of STa and the major subunit ClpG of *E. coli* CS31A fimbriae antigen are secreted across the outer membrane and that the disulfide bonds in the bioactive STa were formed extracellularly by a DsbA-independent mechanism possibly involving molecular oxygen [39,40]. However, these authors also showed that the export of their fusion proteins was dependent on the CS31A secretion pathway and might differ substantially from the secretion of native STa.

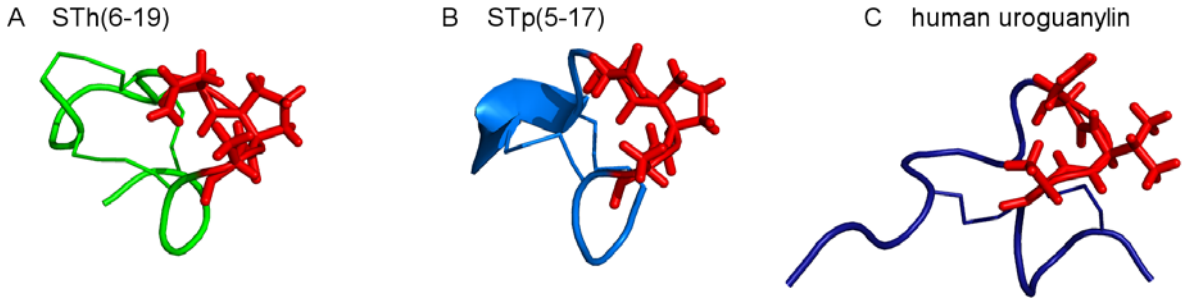
The molecular structure of the toxic domain of STa has been studied by NMR [41] and X-ray crystallography [42–44]. The STp molecule is arranged as a right-handed spiral assembled by three

structural elements: a 3_{10}-helix from Cys5 to Cys9, a type I β-turn from Asn11 to Cys14, and a type II β-turn from Cys14 to Cys17 [42]. This structure is stabilized by the three disulfide linkages as well as by hydrogen bonds formed by the NH groups of most of the constituent amino acids. The latter include inter- as well as intra-turn 4–1 type bonds. The Cys5–Cys10 disulfide linkage adopts a rare right-handed conformation while the other two disulfide linkages have a left-handed spiral conformation [43] which is most common in proteins [45]. All the amino acid side chains are oriented to the outside, giving the molecule a hydrophobic character. The completely exposed β-turn formed by amino acids Asn11, Pro12, and Ala13 [41] is thought to constitute the receptor binding site of STa as point mutations in this region substituting Asn11 by Ala or Ala13 by D-Ala dramatically reduced the receptor binding activity of STa [46]. On the other hand, a Pro12Gly substitution, which is thought to disturb the turn conformation, has only a limited effect on binding activity [47]. In addition, comparative molecular field analysis predicted that the amide backbone of Cys5-Cys6-Glu7-Leu8 plays an important role in the interaction with GC-C [48]. In a recent study, a homohexameric ring-shaped structure of STp(5–17) was observed [44]. In this structure, the interface between adjacent monomers is formed by the 3_{10}-helix of one peptide and the type II β-turn of its neighbor whereas the putative binding site Asn11-Pro12-Ala13 is oriented towards the exterior. Hydrophilic groups are concealed. This gave rise to the suggestion that the hexamer is the native structure recognized by GC-C, however, the hexamer formation may be a result of the hydrophobic solvent chosen in this particular crystallographic study.

Overall, the crystal structure of STp(5–17)[42] and the solution structure of STh(6–19) determined by NMR [41] are in good agreement despite a different orientation of the carboxy termini (Figure 2). Signal broadening of resonances of the amino-terminal residues indicates conformational dynamics in the μs-ms timescale [41,49].

The structure of STa is very similar to the structure of the biologically active A-forms of guanylin and uroguanylin. The additional disulfide bond in STa prevents a similar topological isomerism.

Figure 2. Comparison of (**A**) the solution structure of STh(6–19)[41]; (**B**) the crystal structure of STp(5–17)[42], PDB: 1ETN; and (**C**) the solution structure of human uroguanylin [28], PDB: 1UYA. The hydrophobic region implicated in receptor binding is depicted as red sticks in each structure.



3. The Heat-Stable Enterotoxin Receptor: Intestinal GC-C

The receptor for STa was shown to be intestinal GC-C [50,51]. This 1050 amino acid membrane protein is almost exclusively expressed on the brush border membrane of epithelial cells in the small intestine and the colon [52]. GC-C expression is uniformly distributed along the crypt-villus axis in the

small intestine. However, in the colon, GC-C is mainly restricted to the crypts [53]. The transcription factors CDX2, a caudal family homeodomain protein generally involved in differentiation processes, and hepatocyte nuclear factor-4 (HNF-4), a zinc-finger-containing nuclear hormone receptor, regulate expression of GC-C [54,55]. Besides its function in electrolyte homeostasis, GC-C signaling coordinates proliferation, migration, differentiation and apoptosis in the crypt-villus axis [56].

The primary structure of GC-C [57] indicates that the protein consists of an amino-terminal 23 residue signal peptide, followed by an extracellular domain (ECD), a single transmembrane helix, a kinase homology domain (KHD), a linker region, the actual catalytic guanylyl cyclase domain, and a carboxy-terminal domain [58]. The signal peptide is proteolytically removed during maturation. Other members of the so-called mammalian particulate guanylyl cyclase family include the natriuretic peptide receptors GC-A and GC-B, sensory guanylyl cyclases GC-D, GC-E and GC-F as well as the less studied murine renal GC-G [59]. The latter four GCs have no known ligands and are therefore termed orphan receptors. Whereas the intracellular domains of particulate GCs are closely homologous, the similarity of the extracellular domains is not very pronounced, and, e.g., for GC-C and GC-A the sequence identity is only 19%. A homology model of the ECD of GC-C, based on the known structure of GC-A [60] and experimental data on the configuration of the disulfide linkages, suggests that the ECD consists of two subdomains: one membrane-distal and mainly α-helical and the other one membrane-proximal and predominantly β-sheet type [61]. Indeed, the membrane proximal domain expressed as a single fragment can fold independently [62] and seems to contain the STa binding site [63,64]. The intracellular kinase homology domain plays an important regulatory role. Phylogenetic studies revealed an apparent co-evolution of the KHD and the catalytic domain that also includes the linker region between both domains [65]. The KHD is thought to provide an inhibitory effect on the catalytic domain, which is only relieved by binding of a ligand to the ECD. Consequently, deletion of the KHD results in a constitutive ligand-independent activation of the GC domain [66]. The KHD does bind ATP although it is assumed to be catalytically inactive for lack of the highly conserved H-R-D motif essential for protein kinase activity [67]. The catalytic domain is highly homologous in all particulate as well as the soluble guanylyl cyclases, and it is very similar to the catalytic domain of adenylyl cyclases.

GC-C is a heavily glycosylated protein [68,69] that contains 8–10 *N*-linked glycosylation sites, depending on the species. It is not clear whether the sugar residues are essential for ligand binding. Two differently glycosylated forms of GC-C of 130 kDa and 145 kDa bind STa with the same affinity, although only the mature fully glycosylated 145 kDa GC-C is activated by ligand binding [70]. Since enzymatic deglycosylation of mature GC-C has no effect on binding affinity and activation, the sugar residues might be needed for correct protein folding rather than be directly involved in ligand binding. Moreover, mutagenic removal of specific *N*-glycosylation sites from the extracellular domain of an insect-baculovirus system expressed GC-C [71] resulted in a drop in binding capacity but not in binding affinity, supporting the notion that without certain sugar residues GC-C does not adopt a stable conformation that allows for ligand recognition [72]. However, in the same study, deglycosylation of GC-C by PNGase F resulted in a loss of STa binding.

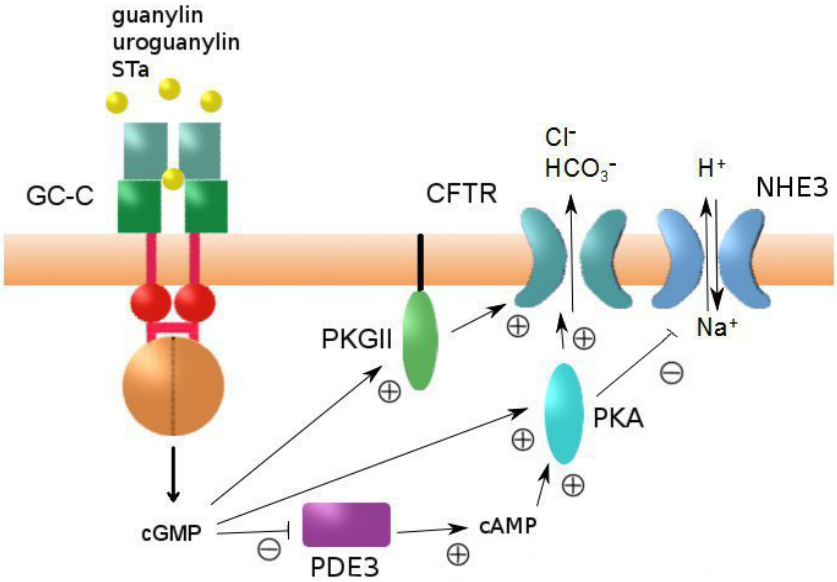
As in adenylyl cyclases, the catalytic domain of GC-C is only active as a dimer. However, whereas adenylyl cylcases and soluble guanylyl cyclases are heterodimers, the active center of particulate guanylyl cyclase is homodimeric with the linker region between KHD and GC domain providing the

necessary dimerization motif. GC-C might actually be present as a homotrimer [73,74] with only two subunits forming the catalytic center on activation. Since the extracellular domains of both GC-A [60] and the homologous atrial natriuretic peptide clearance receptor NPR-C [75] are dimeric, it is possible that the ECD of GC-C also forms oligomers.

4. Regulation of Intestinal Fluid Secretion and STa-Induced Diarrhea

GC-C is a key receptor in regulating the electrolyte level and the fluidity of the intestinal content. Binding of either an endogenous or exogenous ligand to the ECD of GC-C triggers a conformational event, which leads to activation of the catalytic domain and the formation of cGMP. This initiates a signaling cascade that ultimately results in secretion of electrolytes into the intestinal lumen accompanied by water release (Figure 3). Overactivation of GC-C by STa is the physiological basis of ETEC induced watery diarrhea.

Figure 3. Schematic representation of intestinal secretion regulated by GC-C. PDE3: cGMP-inhibitable phosphodiesterase 3; PKGII: cGMP-dependent protein kinase II; PKA: cAMP-dependent protein kinase; CFTR: cystic fibrosis transmembrane conductance regulator; NHE: Na/H exchanger.



The main mediator of chloride secretion is the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel present at the apical membrane of intestinal brush-border epithelial cells [76]. Increased levels of cGMP activate the cGMP-dependent protein kinase II (PKGII), which co-localizes with the CFTR and phosphorylates it [77,78], thus promoting the electrogenic release of Cl^- into the lumen [76]. In addition, cGMP is able to inhibit phosphodiesterase 3 (PDE3). PDE3 hydrolyzes cAMP, and its inhibition results in accumulation of cAMP which, in turn, activates protein kinase A, providing an additional PKGII-independent mechanism to stimulate Cl^- secretion [79]. cAMP as well as cGMP can trigger an increased targeting of CFTR from intracellular vesicles to the cell membrane [80–82].

The Na^+/H^+-exchanger (NHE) is a second target of cGMP action in intestinal epithelia. PKA inhibits the re-absorption of sodium by NHE [83].

Although this mechanism is well established, it was argued recently that water accumulation in the intestinal lumen associated with STa is not as much a result of an active secretion of electrolytes as it is a consequence of impaired fluid absorption [84,85]. It was stressed that the available evidence does not unambiguously point to an increased electrolyte secretion following STa administration.

As main regulator of intestinal fluidity, GC-C is a therapeutic target for treatment of constipation related disorders. Linaclotide, an agonist of GC-C, is closely related to STa and used as an investigational drug against constipation-predominant irritable bowel syndrome (IBS-C) and chronic constipation [86,87].

GC-C and its ligands are also implicated in regulation of the intestinal pH, since CFTR does not only transport Cl^- but also HCO_3^-. Regulation of the pH in the intestine is crucial because a low pH can result in tissue damage. Surprisingly, it was shown that STa can induce HCO_3^- secretion independently of PKGII and CFTR. In CF mice that lack functional CFTR, this secretion is electroneutral and dependent on Cl^-, which suggests involvement of a Cl^-/HCO_3^- exchanger [88]. In GC-C knockout mice an alternative STa receptor is present that causes bicarbonate secretion by a downstream signal cascade different from the PKGII and CFTR mechanism [89].

The possibility of an alternative receptor for STa and guanylin peptides has been investigated very early [90,91]. GC-C knockout mice are not only healthy, fertile and have a normal responsiveness to other secretory signals, they also show statistically significant, albeit strongly reduced, STa binding [92,93]. However, in these animals, no secretion is induced by STa. A non-GC-C, G protein-coupled receptor for guanylin, uroguanylin and STa has been reported in the kidney [94,95].

5. Involvement of GC-C in Colorectal Cancer

Expression of the endogenous GC-C ligands guanylin and uroguanylin is lost in transformed human colon cancer cells [96]. In contrast, GC-C can be found in all types of primary and metastatic colorectal cancer cells [97,98]. Thus, GC-C is a very specific marker for colorectal carcinoma cells that can be exploited for PCR-based tumor detection strategies [99,100]. Due to their very restricted expression outside of intestinal epithelia and their ubiquitous presence on colorectal carcinoma GC-C derived antigens promise to be an important target for immunotherapy of metastatic cancer [101].

Furthermore, its ability to bind GC-C with very high affinity makes STa a useful template structure for synthesis of peptide analogs that target and visualize cancer, for instance by CT imaging [102–107]. In addition, the potential use of structurally less demanding uroguanylin analogs has been investigated [108].

Since STa is internalized upon binding of GC-C in T84 cells, it might be used to deliver diagnostic or therapeutic drugs into colorectal carcinoma cells [109].

Recently, it has been hypothesized that STa is not only suitable for efficient targeting of colorectal carcinoma cells but may also prevent proliferation of these cells and thus inhibit initiation and progression of colon cancer [110]. Indeed, an inverse relationship between the global incidence of colorectal cancer and the ETEC infection frequency can be found. While colorectal cancer is a common cause of mortality in Western societies it is rather rare in developing countries [111]. This striking observation together with the apparent ability of STa and uroguanylin to delay the progression of the cell cycle in human colon cancer cells via a cGMP- and Ca^{2+}-dependent pathway [110,112,113] lead to a new view on the events involved in colorectal tumorigenesis. It was speculated that lack or

insufficient expression of the paracrine hormones guanylin and uroguanylin affecting GC-C and its downstream effectors are at the very beginning of cancer formation in intestinal cells [114]. As mentioned above, the guanylin peptides are frequently lost in very early stages of colorectal carcinogenesis. Inversely, their receptor GC-C is even overexpressed in colorectal tumors, probably in order to compensate hormone deficiency [115]. GC-C's transcriptional regulator CDX2 is overexpressed in certain forms of colorectal cancer as well [116]. At first, this discovery seemed to question the role of both proteins as tumor suppressors, but *in vivo* experiments with mice showed that elimination of the two proteins promotes both tumor initiation and growth [117]. Furthermore, differential microarray analysis revealed that interruption of GC-C signaling leads to activation of pro-oncogenic signaling by the AKT pathway [118]. Thus, the mechanism underlying GC-C's function as a tumor suppressor seems to be the control of proliferation and metabolism of the intestinal epithelium in order to maintain genetic stability [119]. As disruption of GC-C signaling in the earliest stages of colorectal cancer is a consequence of hormone insufficiency, it is to be assumed that hormone replacement therapy may be able to prevent or treat colon cancer. Actually, feeding of uroguanylin to $Apc^{Min/+}$ mice, an animal model for colon cancer, leads to a reduction of both tumor number and size [120]. Being an agonist of uroguanylin and guanylin, STa also results in inhibition of proliferation in human colorectal cancer cells [110,112,121].

6. Outlook

The previous paragraphs underline that recent research has helped to transform STa's reputation from that of a curse torturing or even killing thousands of people in developing countries, to that of a cure for less severe as well as life-threatening diseases. STa analogs for treatment of constipation are already in the late phase of clinical trials and uroguanylin as well as different STa analogs that target colon cancer therapy might soon follow.

However, a reliable cure for acute secretory diarrhea elicited by STa is still missing. One reason for this might be the lack of structural and mechanistic information as to STa receptor interaction, and such data on GC-C and the GC-C:STa complex would be extremely valuable. Consequently, an important step towards the development of a remedy against STa mediated diarrhea would be the determination of the three-dimensional structure of the ligand-binding extracellular domain of GC-C. Comparison of the structures in absence and in presence of STa could help to elucidate the activation mechanism. These data could pave the way to the design of either antagonistic STa analogs blocking the receptor or scavengers competing with it for STa binding.

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