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STa Peptide Analogs For Probing Guanylyl Cyclase C

Xiaobing Tian^{1,†}, Allison M. Michal¹, Peng Li², Henry R. Wolfe^{2,‡}, Scott A. Waldman^{2,3}, and Eric Wickstrom^{1,3,*}

¹Department of Biochemistry & Molecular Biology, Thomas Jefferson University, Philadelphia PA 19107

²Department of Pharmacology & Experimental Therapeutics, Thomas Jefferson University, Philadelphia PA 19107

³Kimmel Cancer Center, Thomas Jefferson University, Philadelphia PA 19107

Abstract

Guanylyl cyclase C (GCC), universally over-expressed on primary and metastatic colorectal carcinoma cells, is activated by endogenous ligands guanylin and uroguanylin and exogenous 18 residue heat-stable enterotoxins (STa) produced by diarrheagenic bacteria. Two 12 residue STa analogs with different combinations of two interlocked disulfide bonds, peptide **3** and peptide **6**, were synthesized by orthogonal solid phase synthesis routes. Peptide **3** and peptide **6** bound GCC with a rank order potency of STa > peptide **3** > peptide **6**. Peptide **6** behaved as an agonist. Conversely, peptide **3** was an antagonist of GCC at nM concentrations, but an agonist at μ M concentrations. These observations demonstrate the utility of this novel solid phase synthesis route for generating peptides with complex disulfide bonds. Furthermore, the results reveal that the minimum toxic domain of ST encompasses 12 amino acid residues. Moreover, they identify a specific sequence of this diarrheagenic toxin that serves at low concentration as an antagonist for GCC.

Keywords

colorectal cancer; oncogenes; peptides

INTRODUCTION

Colorectal cancer is the third most common neoplasm and the second leading cause of cancer-related mortality in the United States, responsible for 10% of cancer-related deaths¹. While computerized tomography (CT), the imaging modality of choice for this disease, detects metastases 5 mm in diameter², it is not sufficient to detect metastases during staging, monitoring of established disease progression, or post-operative surveillance for disease recurrence². Similarly, although positron emission tomography (PET) of 2'-[¹⁸F]fluorodeoxyglucose (FDG) uptake by rapidly metabolizing cells detects recurrent disease and identifies resectable metastases³, it is not routinely applied to the evaluation of colorectal cancer patients³.

CORRESPONDING AUTHOR FOOTNOTE: Dr. Eric Wickstrom, Department of Biochemistry & Molecular Biology, Thomas Jefferson University, Philadelphia PA 19107-5541, voice: 1-215-955-4578; fax: 1-215-955-4580, eric@tesla.jci.tju.edu; website: <http://tesla.jci.tju.edu>.

[†]Current address: Department of Medicine (Hematology/Oncology), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

[‡]Current address: Genaera Corporation, Plymouth Meeting, Pennsylvania 19462

Guanylyl cyclase C (GCC) is normally expressed in apical membranes of intestinal mucosal cells but not in extra-intestinal tissues or tumors. However, GCC is over-expressed by, and serves as a highly specific marker for, primary and metastatic colorectal carcinoma cells^{5,6}. GCC is a receptor activated by the endogenous ligands guanylin and uroguanylin, and the exogenous ligand produced by *E. coli*, heat-stable enterotoxin (STa), an 18 amino acid peptide that causes secretory diarrhea in animals and humans⁷. We hypothesize that imaging of guanylyl cyclase C receptor (GCC) over-expression might improve the sensitivity and specificity of colorectal cancer detection⁴.

The three intrachain disulfide bridges of STa (Fig. 1) are critical for heat stability, and binding and activating GCC⁸. Binding of STa to the extracellular ligand binding domain of GCC activates the cytoplasmic guanylyl cyclase catalytic domain, producing cGMP from GTP. Subsequently, cGMP activates protein kinases which phosphorylate the cystic fibrosis transmembrane conductance regulator, a chloride channel, stabilizing its open conformation⁹, resulting in net fluid and electrolyte secretion and diarrhea. Moreover, activation of GCC induces receptor-mediated endocytosis and internalization of ligand-receptor complexes¹⁰.

Peptide analogs of insulin-like growth factor 1 have been employed to deliver a peptide nucleic acid (PNA) *KRAS* hybridization probe into pancreas cancer xenografts, enabling SPECT and PET imaging of *KRAS* mRNA¹¹. Because GCC is a specific target for colorectal tumors, analogs of STa might deliver diagnostic and therapeutic cargo into colon cancer cells through GCC-dependent endocytosis. Studies of structure-activity relationships for STa demonstrated that the 13 amino acid domain from Cys5 to Cys17 retains full enterotoxigenic activities, wherein the three intramolecular disulfide bonds between Cys5 and Cys10, Cys6 and Cys14, and Cys9 and Cys17 each modulate binding affinity⁸. Moreover, while, a minimum of two disulfide bridges are required for ST binding to GCC, that bond between Cys6 and Cys14 is absolutely required¹². Here, we report the synthesis and characterization of truncated STa analogs with two specific disulfide bridges that bind to GCC.

MATERIALS

General

Reagents and solvents were purchased from Sigma-Aldrich (St. Louis MO), except for Ellman's reagent, 5,5'-dithio-bis(2-nitrobenzoic acid) (Uptima, Montlucon, France), and Fmoc-L-amino acid derivatives and NovaSyn TGR resin (0.2 – 0.3 mmol/g) (NovaBiochem, San Diego CA).

Synthesis and Analysis

Linear peptides were assembled on an Applied Biosystems 430A peptide synthesizer by standard Fmoc coupling. Preparative and analytical HPLC were performed with an Alltima 10×250 mm C₁₈ 5 μ reverse phase column (Alltech Associates, Deerfield IL), or a Microsorb 10×250 mm C₁₈ 5μ column (Varian Associates, Palo Alto CA) on a 600E liquid chromatograph (Waters Associates, Milford MA) using manual injection. The crude aqueous phase was separated on a 10×250 mm Alltech Alltima C₁₈ (5 μ) column eluted with a 40 min gradient from 14.25% CH₃CN to 33.25% CH₃CN in aqueous 0.1% CF₃CO₂H at 1 mL/min at 25°C, monitored at 220 nm.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out from an α-cyano-hydroxycinnamic acid matrix excited at 338 nm on a SELDI (Ciphergen, Fremont, CA) calibrated with porcine neuropeptide Y. Electrospray ionization mass spectrometry (ESI-MS) was carried by automatic injection of unknown in

aqueous 0.1% CF₃CO₂H on a SciEx API3000 MS/MS (Applied Biosystems, Foster City CA).

STa Binding

Mouse intestinal mucosal cells containing GCC were isolated¹³, and diluted to a final concentration of 0.5–2.0 mg protein/mL. Millipore 96-well plates with MAHVB membrane filters were incubated with 0.3% polyethylenimine solution overnight. Plates were then washed 6 times with 200 μ L of washing buffer (23.64 g Tris-HCl, 78.89 g NaCl, 3 g bacitracin, 1.11 g EDTA, 800 mL double distilled water). Each well received 50 μ L 3 $\times$ binding buffer (23.64 g Tris-HCl, 0.44 g cysteamine, 78.89 g NaCl, 3 g bacitracin, 1.11 g EDTA, 800 mL double distilled water), 40 μ L of double distilled water, 40 μ L of intestinal membranes, 10 μ L of 60,000 cpm [¹²⁵I]STa (2000 Ci/mmol)¹³ and 10 μ L of the indicated concentration of STa or its analogs. Plates were incubated overnight at 4°C and then washed 6 times with 200 μ L of washing buffer. Membranes were punched into pre-labeled 12 $\times$ 75 borosilicate glass tubes and radioactivity quantified a Packard 5000 gamma spectrometer (Packard Instruments, Meriden CT). Data were analyzed using Prism Graph Pad and K_i calculated using the Cheng-Prusoff equation¹⁴.

Cyclic GMP

T84 human colon cancer cells were grown to confluence in 24-well plates with DMEM supplemented with 2 mM glutamine, 5000 U/mL penicillin, 50 μ g/mL streptomycin, 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) at 37°C in a humidified incubator containing 5% CO₂/95% air. Cells were washed 3 times with 500 μ L of PBS and incubated for 30 min with isobutylmethylxanthine (IBMX) at 37°C. Cells were stimulated with 10 μ L of controls, PBS or 1.0 μ M STa, or 10 μ L of the indicated concentration of STa analog for 15 min at 37°C. Reactions were stopped and cells lysed by adding 50 μ L of aqueous 12% CCl₃CO₂H and vortexing. Lysates were sedimented at 12,000 $\times$ g for 15 min at 4°C. Supernatants were separated from pellets and saved. Pellets were suspended in 500 μ L of 0.1 NaOH, and used to quantify protein.

Cyclic GMP was analyzed by radioimmunoassay as described¹⁵. Cyclic GMP in duplicate 100 μ L aliquots of each supernatant was acetylated by adding 20 μ L of Et₃N and 10 μ L of Ac₂O, followed by to 100 μ L of 20 mM CaCl₂ in 50 mM NaOAc, pH 4.0. 100 μ L of anti-cGMP polyclonal antibody and 100 μ L of [¹²⁵I]cGMP (20,000 cpm)¹³ were then added to each tube followed by overnight incubation at 4°C. On the following day, 100 μ L of 1 mg/mL γ -globulin and 2 mL of cold 2-PrOH were added to all standard and sample tubes. Tubes were sedimented at 15,000 $\times$ g for 30 min at 4°C. Supernatants were decanted and pellets dried, and radioactivity quantified in a Packard 5000 gamma spectrometer (Packard Instruments, Meriden CT).

RESULTS

Design and synthesis of the STa analogs for GCC receptors

Peptide **3** includes the 13 residue toxin domain of STa with two disulfide bridges between Cys6 and Cys14, and Cys5 and Cys10, respectively, where Cys9 and Cys17 are replaced by two Ala residues to eliminate the third disulfide bridge (Fig. 1). Peptide **6** includes a 12 residue toxin domain with two disulfide bonds between Cys6 and Cys14, and Cys9 and Cys17, respectively, where Cys5 is eliminated, and Cys10 is replaced by Ala to eliminate the first disulfide bridge (Fig. 1). Prior syntheses of STa analogs required simultaneous disulfide bond formation of fully protected peptide by air-oxidation in aqueous solutions¹⁶, producing complex mixtures, including misfolded peptides reflecting incorrect intramolecular cyclization, such as discrete and nested disulfide bonds, and byproducts

resulting from intermolecular formation of disulfide bonds¹⁷. Moreover, these syntheses require peptide purification followed by bioassay to identify desired products¹⁷. Here, orthogonal formation of two interlocked disulfide bonds in STa analogs ensured unambiguous formation of disulfide bridges. Thus, acetamidomethyl (Acm) and trityl (Trt) are orthogonal Cys thiol protection groups and simultaneous cleavage and removal of trityl, cyclization with air or Me₂SO, followed by I₂, selectively directed the orderly formation of two disulfide bridges¹⁸.

Synthesis of peptide 3

Peptide **3** was assembled by solid phase Fmoc coupling, and then cleaved from the resin with 95% CF₃CO₂H/2.5% CH₃SC₆H₅/2.5% H₂O at room temperature for 2 h. Initially, cysteines were cyclized by air oxidation¹⁹, assuming that the smaller loop would be easiest to form. Five μ mol (6.48 mg) of peptide **1** with deprotected Cys5 and Cys10 were dissolved in 10 mL of 0.01 M Na₂HPO₄, pH 8.0, plus 200 μ L Me₂SO. However, RP-HPLC and MALDI-TOF did not reveal new compounds after stirring for 3 d at room temperature, even with addition of 2% Me₂SO to increase oxidation¹⁹, suggesting strong steric hindrance with formation of the Cys5-Cys10 disulfide. Conversely, the large loop between Cys6 and Cys14 was cyclized first (Fig. 2) by stirring 21.07 μ mol (27.30 mg) crude peptide **1** with deprotected Cys6 and Cys14 in 10 mL of 0.01 M Na₂HPO₄, pH 8.0, plus 200 μ L Me₂SO for 24 h. At 24 h, a new RP-HPLC peak having a longer retention time than peptide **1** appeared. After 72 hrs, the starting material disappeared, without free thiols²⁰ indicating the efficient formation of the Cys6-Cys14 disulfide. Products were concentrated, purified by HPLC, lyophilized to a yield of 48.1% pure peptide **2**, with a mass by MALDI-TOF MS of 1364.4 Da (calculated 1364.6 Da) and M+Na⁺ (1386.1 Da).

The Cys5-Cys10 disulfide was formed (Fig. 2) by stirring 9.52 μ mol peptide **2** (13.1 mg) in 6.3 mL of 50% Me₂SO/50% HOAc, pH to 4–4.5, with 47.6 μ mol I₂ (12.1 mg) for 30 min. Saturated aqueous ascorbic acid was dropped into the solution to remove excess I₂. The mixture was extracted with Et₂O (4 mL) three times, until the organic layer was colorless. The aqueous layer was then evaporated under vacuum to 2 mL, removing residual ethanol, and an additional 5 mL of H₂O was added. The resulting peptide **3** purified by RP-HPLC at 23.35 min (Fig. 3) produced 7.4 mg of white powder with a yield of 63.4% and a mass by MALDI-TOF of 1219.6 Da (calculated 1220.46 Da) (Fig. 4). Here, rapid cyclization of the second disulfide implied that formation of the larger loop facilitated formation of the smaller loop by approximating Cys5 and Cys10.

Synthesis of peptide 6

Peptide **6** was assembled by solid phase Fmoc coupling, and then cleaved from the resin with 95% CF₃CO₂H/2.5% (iPr)₃SiH/2.5% H₂O at room temperature for 2 h. Mass spectroscopy of peptide **1** (see above) revealed product that had lost one Acm thiol protecting group reflecting cleavage by thioanisole and, therefore, triisopropylsilane [(iPr)₃SiH], a highly sterically hindered agent with less potential to remove Acm groups, scavenged free radicals in the cleavage cocktail. The Cys6-Cys14 disulfide was formed by stirring 7.7 μ mol of peptide **4** (10 mg) with deprotected Cys6 and Cys14 in 10 mL of Me₂SO:H₂O (1:2) at room temperature for 4 h (Fig. 5; peptide **5**). Since disulfide formation by air oxidation typically performed under basic conditions (pH 7.5–9.0) is incompatible with coupling to PNAs, which precipitate at pH >7, disulfide formation was optimized using Me₂SO/67% H₂O at room temperature¹⁸. At 4 and 24 h, ESI-MS (Fig. 6A, B) revealed a peak of 1293.7 Da, the mass of peptide **5**, without free thiols²⁰, indicating the efficient formation of the Cys6-Cys14 disulfide. It is noteworthy that the time for cyclizing the first disulfide in this one-pot reaction was reduced from 3 d for peptide **3**, to 4 h for peptide **6**. Oxidation/cyclization of Acm-protected residues for the Cys5-Cys10 disulfide required

acidic conditions and the reaction was adjusted to pH to ~4 with 10 mL HOAc, followed by stirring with 36.2 μ mol I₂ (9.2 mg) at room temperature. ESI-MS (Fig. 6C) revealed a peak of 1149.6 Da (calculated 1149.5 Da), without free thiols²⁰, in <1 h indicating the efficient formation of peptide **6**. Excess I₂ was removed by saturated aqueous ascorbic acid, HOAc by Speed Vac (Savant, Holbrook NY) followed by extraction with EtOH (5 mL) three times, until the organic layer was colorless. Peptide **6** was purified by HPLC (Fig. 7), yielding a peak at 21.65 min containing 3.72 mg white powder with a yield of 41.9%.

Functional analysis of peptides **3** and **6**

Binding to GCC was assessed by quantifying competitive displacement of [¹²⁵I]STa from GCC expressed in membranes isolated from mouse colon. STa displaced [¹²⁵I]STa in a concentration, graded and saturable fashion (Fig. 8A) with a K_i of 1.5 $\pm$ 0.6 nM (Table 1), in close agreement with previous estimates of 0.1 nM-1.0 nM K_d⁹. Similarly, peptides **3** and **6** displaced [¹²⁵I]STa in a concentration graded and saturable fashion (Fig. 8B,C) with K_i's of 30.6 $\pm$ 13.0 nM and 57.6 $\pm$ 23.1 nM, respectively (Table 1), establishing the rank order potency of the affinity of these peptides as: STa > peptide **3** > peptide **6**.

Beyond receptor binding, synthetic peptides were analyzed for their ability to activate the catalytic domain of GCC by quantifying accumulation of cGMP in T84 human colon carcinoma cells. Peptide **3** at 500 nM or below had no effect on intracellular cGMP but displayed agonist activity above 500 nM (Fig. 9A). Furthermore, peptide **3** up to 10⁴ nM (10 μ M) blocked accumulation of cGMP in T84 cells induced by STa (Fig. 9C). Peptide **6**, on the other hand, induced cGMP accumulation in a concentration-dependent fashion, maximally increasing intracellular cGMP ~50-fold (Fig. 9B).

DISCUSSION

The focus here is to define peptide analogs of STa, a natural ligand for GCC that is a highly specific target for colorectal cancer, that can deliver PNAs to image gene expression during neoplastic transformation in intestinal cells. Here, an orthogonal synthesis of STa analogs with different combinations of two interlocked disulfide bonds produced peptides with a rank order potency for receptor ligation of STa > peptide **3** > peptide **6**. These analyses demonstrate that the binding domain of STa can be reduced to 12 amino acid residues.

The binding affinities of peptides **3** and **6** are sufficient to support *in vivo* targeting of GCC for colon cancer imaging. However, to deliver PNA probes specifically into colon cancer cells for binding to mRNA, ligand-receptor interaction requires productive activation of downstream signaling events to initiate internalization¹⁰. Conversely, analogs that fail to activate downstream events and antagonize receptor activation by native ligands may not undergo internalization and delivery into cells. Here, engagement of the receptor domain by peptide **6** activated the catalytic domain of GCC, inducing accumulation of intracellular cGMP in T84 human colon cancer cells. This agonist activity of peptide **6** was analogous to STa but of lower potency, appropriately reflecting the rank order of receptor affinities. Engagement of the receptor binding domain of GCC with productive activation of downstream signaling events suggest that peptide **6** will be suitable for delivery of PNA probes for molecular imaging of gene expression in colon cancer cells.

By contrast, physiologically relevant concentrations of peptide **3** engaged the GCC receptor domain, displacing STa from binding, but did not activate the catalytic domain or induce cGMP accumulation in T84 cells. The failure to induce accumulation of cGMP in these cells by peptide **3** at low concentrations did not reflect anomalous activation of phosphodiesterases, which degrade cyclic nucleotides, since these studies were performed with IBMX, a potent inhibitor of phosphodiesterases. Moreover, peptide **3** opposed the

ability of STa to activate the catalytic domain of GCC and induce intracellular accumulation of cGMP. We hypothesize that naturally occurring proteases might be responsible for the appearance of agonist activity by peptide **3** at high concentrations.

Receptor engagement without activation of downstream effector mechanisms that oppose activation of those mechanisms by native ligands suggests that peptide **3** at physiologically relevant concentrations fulfills criteria for a GCC receptor antagonist. In the context of the current studies, these observations suggest that peptide **3** would not be particularly suitable for delivery of diagnostic or therapeutic payloads into colon cancer cells through GCC, reflecting the likely inability of this analog to induce receptor internalization.

While cancer is a major cause of morbidity and mortality in the developed world, infectious diarrhea is the second leading single cause of loss of quality-adjusted life years worldwide. Although there are many infectious agents that induce diarrheal disease, ~33% of cases reflect infections with enterotoxigenic *E. coli*. These organisms secrete a number of enterotoxins that induce diarrhea, however, ~70–80% of isolates from humans secrete heat-stable enterotoxin (STa's). Indeed, STa's are a leading cause of diarrheal disease in animals and humans worldwide. In that context, peptide **3** antagonizes activation of downstream signaling events induced by activation of GCC by STa. The synthesis described here is the first to produce an analog of STa that functions as an antagonist for GCC at physiological concentrations. These observations suggest that peptide **3** might serve as a lead structure for the development of GCC antagonists to prevent and treat diarrheal disease induced by enterotoxigenic *E. coli* secreting STa. Beyond antagonizing downstream signaling events induced by STa's mediated by GCC, the ability of peptide **3** to prevent the pathophysiological consequences of infection by enterotoxigenic *E. coli* secreting STa's and the associated induction of diarrheal disease will require further study in animal models of secretory diarrhea.

SUMMARY

The syntheses described here have produced analogs of STa with potential diagnostic and therapeutic utility across a spectrum of disease. They are noteworthy for simplifying the synthesis of these complex 13 residue three disulfide peptides, revealing the receptor activity of peptides with 12 residues and two disulfides which can be generated in 4 h rather than 3 d. The present studies predict that radiolabeled peptide **6** will have utility for *in vivo* colon cancer imaging by targeting GCC⁴. Also, a therapeutic cargo could be linked to peptide **6** for specific delivery into colorectal cancer cells directed to GCC. Furthermore, it is possible that a PNA hybridization probe conjugated to peptide **6** could be internalized by colorectal cancer cells for oncogene mRNA imaging²². The latter approach requires an assay for receptor-mediated endocytosis to verify that the full length probe is internalized by colorectal cancer cells and the PNA binds to target mRNA for visualization.

Conversely, peptide **3** is an antagonist of GCC at nM concentration, but an agonist at μ M concentration. Thus, peptide **3** could be a starting point for the design of a molecule for treating and preventing enterotoxigenic bacterial diarrhea. The translation of these observations into clinical utility in patients with cancer and infectious diarrhea is a principal focus for future studies.

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Figure 1.
The structure of STa and design of STa analogs of peptide **3** and peptide **6**.

Figure 2.

Synthesis of peptide **3**, performed as described in Methods and Results.

Figure 3.

Preparative HPLC of crude peptide **3** after formation of the second disulfide bridge. The crude aqueous phase was applied to a 10×250 mm Alltech Alltima C₁₈ (5μ) column eluted at 25°C with a 40 min. gradient from 14.25% CH₃CN to 33.25% CH₃CN in aqueous 0.1% CF₃CO₂H at 1 mL/min, monitored at 220 nm, shown on the left y-axis. % CH₃CN (blue line) is shown on the right y-axis.

Figure 4. MALDI-TOF mass spectrum of peptide **3** in positive mode, desorbed and ionized from a matrix of α -cyano-4-hydroxycinnamic acid. Peptide **3** appears as a molecular ion peak ($M + H^+$) of 1219.6 Da (calculated 1220.46 Da).

Figure 5.
Synthesis of peptide **6**, performed as described in Methods and Results.

Figure 6.

The progress of one-pot orthogonal disulfide bond formation of peptide **6**. **A:** ESI-MS of the first cyclization reaction after 4 hr, showing a clear main peak of 1293.7 Da, the molecular mass of peptide **5**. **B:** ESI-MS of the first cyclization reaction after 24 hr, showing a clear main peak of 1293.7 Da, the molecular mass of peptide **5**. **C:** ESI-MS of the second cyclization reaction after 1 hr, showing a clear main peak of 1149.6 Da, the molecular mass of peptide **6**.

Figure 7.

Preparative HPLC of crude peptide **6** after formation of the second disulfide bridge. The crude aqueous phase was applied to a 10×250 mm Varian Microsorb C₁₈ (5μ) column eluted at 25°C with a 30 min. gradient from 11.4% CH₃CN to 47.5% CH₃CN in aqueous 0.1% CF₃CO₂H at 2 mL/min, monitored at 210 nm, shown on the left y-axis. % CH₃CN (blue line) is shown on the right y-axis.

Figure 8.

Competitive displacement of $[^{125}\text{I}]\text{STa}$ binding to GCC in T84 human colon carcinoma cell membranes by synthetic peptides as outlined in Methods. **A:** synthetic STa; **B:** peptide 3; **C:** peptide 6. Data represent triplicate measurements from three independent experiments.

Figure 9.

Stimulation of cGMP production by T84 cells. The ability of peptide **3** (A) and peptide **6** (B) to induce cGMP accumulation in T84 human colon cancer cells was quantified as described in Methods. C: The ability of peptide **3** to antagonize GCC activation by STa was quantified by pre-incubating T84 human colon carcinoma cells for 30 min at 37°C with the indicated concentration of peptide **3**, followed by addition of 30 nM STa. Incubations were continued for 15 min. cGMP accumulation was quantified by radioimmunoassay as described in Methods. Data represent duplicate measurements from two independent experiments.

Table 1EC₅₀ and K_i Values for STa Analogs

Competitor	EC ₅₀ , nM ^a	K _i , nM ^b
STa	5.1±1.2	1.5±0.6
peptide 3	111.3±1.3	30.6±13.0
peptide 6	229.8±2.0	57.6±23.1

^aEC₅₀ values ± standard error of the means were measured by displacement of [¹²⁵I]STa from GCC on mouse intestinal cells¹³.

^bValues of K_i were calculated using the Cheng-Prusoff equation¹⁴.