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# Bioactivity of Oral Linaclotide in Human Colorectum for Cancer Chemoprevention.

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## Research Article

### Bioactivity of oral linaclotide in human colorectum for cancer chemoprevention

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**Abbreviations:** AOM, azoxymethane; CFTR, cystic fibrosis transmembrane conductance regulator; cGMP, cyclic GMP; EIA, enzyme-linked immunoassay; ETEC, enterotoxigenic *E. coli*; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GUCA2A, guanylin; GUCA2B, uroguanylin; GUCY2C, guanylyl cyclase C; PD, pharmacodynamic; PEG, polyethylene glycol 3350; PKG, cGMP-dependent protein kinase; SD, standard deviation; STs, bacterial heat-stable enterotoxins; TCA, trichloroacetic acid; VASP, vasodilator stimulated phosphoprotein.

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## Competing Interests

S.A. Waldman is the Chair of the Data Safety Monitoring Board for the Chart-1 Trial™ sponsored by Cardio3 Biosciences, and the Chair (uncompensated) of the Scientific Advisory

Board of Targeted Diagnostics & Therapeutics, Inc., which provided research funding that, in part, supported this work and has a license to commercialize inventions related to this work. S.A. Waldman is the Samuel MV Hamilton Professor of Thomas Jefferson University. ESB and DJM received F30 Ruth Kirschstein MD-PhD Fellowship Awards.

## **Abstract**

Guanylate cyclase C (GUCY2C) is a tumor suppressing receptor silenced by loss of expression of its luminocrine hormones guanylin and uroguanylin early in colorectal carcinogenesis. This observation suggests oral replacement with a GUCY2C agonist may be an effective targeted chemoprevention agent. Linaclotide is an FDA approved oral GUCY2C agonist formulated for gastric release, inducing fluid secretion into the small bowel to treat chronic idiopathic constipation. The ability of oral linaclotide to induce a pharmacodynamic response in epithelial cells of the colorectum in humans remains undefined. Here, we demonstrate that administration of 0.87 milligrams of oral linaclotide daily for 7 days to healthy volunteers, after oral colon preparation with polyethylene glycol solution (MoviPrep), activates GUCY2C, resulting in accumulation of its product cyclic (c)GMP in epithelial cells of the cecum, transverse colon, and distal rectum. GUCY2C activation by oral linaclotide was associated with homeostatic signaling, including phosphorylation of vasodilator-stimulated phosphoprotein and inhibition of proliferation quantified by reduced Ki67-positive epithelial cells. In the absence of the complete oral colonoscopy preparation, linaclotide did not alter cGMP production in epithelial cells of the colorectum, demonstrating that there was an effect related to the laxative preparation. These data show that the current FDA-approved formulation of oral linaclotide developed for small bowel delivery to treat chronic idiopathic constipation is inadequate for reliably regulating GUCY2C in the colorectum to prevent tumorigenesis. The study results highlight the importance of developing a novel GUCY2C agonist formulated for release and activity targeted to the large intestine for colorectal cancer prevention.

## Introduction

Colorectal cancer is the 4th most commonly diagnosed cancer in the United States, with approximately 150,000 new cases recorded each year.(1) Over the course of a lifetime, about 1 in 20 U.S. residents will be diagnosed with this disease. Despite advances in early detection and treatment, the mortality rate for colorectal cancer remains nearly 50%. Although screening and surveillance continue to be the cornerstone of colorectal cancer prevention, chemoprevention has emerged as a complementary approach among higher risk participants. To date, aspirin (ASA) and other nonsteroidal anti-inflammatory drugs (NSAIDs) represent the most thoroughly investigated class of colorectal cancer chemoprevention agents. However, given the established risk/benefit profile of these agents, the widespread use of ASA or other NSAIDs strictly for colorectal cancer chemoprevention seems unlikely in the average-risk population.

Guanylate cyclase C (GUCY2C) is the intestinal epithelial cell receptor (2) for the endogenous hormones guanylin and uroguanylin. Hormone-receptor interaction activates the intracellular catalytic domain, which converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP). This cyclic nucleotide activates signaling intermediates, including cGMP-dependent protein kinase (PKG), which phosphorylates downstream effectors including vasodilator-stimulated phosphoprotein (VASP) and cystic fibrosis transmembrane conductance regulator (CFTR). Phosphorylation of CFTR opens this chloride channel, resulting in fluid and electrolyte secretion. This mechanism has been co-opted by bacteria that secrete heat-stable enterotoxins (STs), which are structural and functional homologs of guanylin and uroguanylin, to induce GUCY2C-dependent diarrhea.(3-5) Beyond secretion, GUCY2C and its ligands also regulate intestinal homeostasis along the crypt-villus axis by restricting proliferative dynamics and

coordinating cell cycle, differentiation, and metabolic circuits.(6-8) In that context, guanylin and uroguanylin are the most commonly lost gene products in colorectal cancer in animals and humans.(9-11) Of significance, epithelial cells undergoing transformation continue to express GUCY2C. Indeed, colon cancer cells over-express GUCY2C compared to normal adjacent mucosa.(12, 13) Moreover, we have previously demonstrated that pharmacologic or genetic delivery of GUCY2C ligands opposes intestinal tumorigenesis in mice.(14, 15)

Taken together, these data support that GUCY2C is a tumor suppressing receptor when silenced, due to the loss of expression of guanylin and uroguanylin, universally contributes to early development of colorectal cancer. These properties highlight the potential value of oral replacement with GUCY2C agonists as targeted prevention for colorectal cancer. Oral GUCY2C agonists have impressive safety profiles in pre-clinical through late-stage clinical trials for the treatment of chronic constipation syndromes. Given the paucity of compounds proven safe and effective for colorectal cancer chemoprevention, this class of agent warrants further investigation. Linaclotide is an FDA approved GUCY2C agonist formulated for immediate gastric release, with bioactivity in the small intestine. It is approved for the treatment of irritable bowel syndrome with constipation and for chronic idiopathic constipation. The chemopreventive-relevant pharmacodynamic response of linaclotide in the human colon was not assessed during the agent's development. Here, we evaluated the effects of linaclotide in epithelial cells of the colorectum in healthy volunteers.



## Materials and Methods

### Study Design

The study was designed to test the hypothesis that orally administered linaclotide (Ironwood Pharmaceuticals, Inc., Cambridge, MA) engaged GUCY2C in the colorectum. This study was important because the current formulation of linaclotide was designed to treat chronic constipation by releasing the bioactive peptide in the stomach's acidic environment, which stimulates fluid secretion in the proximal small bowel. In its current formulation, only ~1-3% of orally administered linaclotide or its active metabolite is recovered in stool.(16) The present study examined whether sufficient concentrations of the orally administered peptide can successfully engage GUCY2C in epithelial cells of the colon and rectum, key targets for chemoprevention. The study comprised three stages (Fig. 1). In Stage I, we evaluated the ability of a single oral daily dose of 0.87 mg of linaclotide administered for seven days to activate cGMP production in the colon and rectum sampled by colonoscopic biopsy following oral bowel preparation. Stage II explored the ability of that same dose to activate rectal GUCY2C (the most distant site for chemoprevention) by sigmoidoscopy sampling following oral bowel preparation. In Stage III, we explored the ability of linaclotide 0.87 mg to activate GUCY2C in rectal mucosa. Biopsies were obtained by sigmoidoscopy following rectal preparation by tap water or PEG enema. Stage III was designed to determine if the orally administered colonic bowel preparation affected the colonic distribution of linaclotide. We anticipated that successful completion of these three stages would offer a dose reduction employing sigmoidoscopy and distal bowel cleansing by enema to identify the optimal dose of linaclotide for a subsequent chemoprevention trial.

The study was approved by the IRBs of the Mayo Clinic, Thomas Jefferson University, and Fox Chase Cancer Center and registered on ClinicalTrials.gov (NCT01950403). The study population included healthy volunteers 18-65 years old, without personal or first degree family history of CRC, inflammatory bowel disease or other recent ( $\leq 3$  months prior to day 0) or ongoing diseases producing acute or chronic diarrhea. In Stage I, subjects received oral bowel preparation with 100 grams of polyethylene glycol 3350-electrolyte solution (PEG) (MoviPrep™, Salix Pharmaceuticals) followed by a screening colonoscopy. Only subjects who tolerated the anesthesia and bowel preparation and who had no significant intestinal pathology were eligible to proceed to the intervention phase of the study (Fig. 1). For the intervention phase, participants were randomly assigned to receive a single oral dose of either placebo or linaclotide 0.870 mg daily following an overnight fast for 7 consecutive days. To assure compliance, subjects returned each day to the clinical research unit for witnessed dosing. On day 7, participants received the second dose of MoviPrep at approximately 3:30 AM, and the final oral dose of linaclotide or placebo 2 h later. Participants underwent the second colonoscopy 8 hours after the final linaclotide/placebo dose. A total of 24 biopsies were taken from each participant during the screening and again during the post-intervention colonoscopies; 8 from each of the 3 anatomical locations including cecum, transverse colon, and rectum. Three samples from each anatomical site were flattened immediately and fixed in pre-chilled paraformaldehyde (4%) overnight followed by standard tissue processing for analysis of Ki67. The remaining five samples from each anatomical location were flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for analysis of cGMP levels (2 samples) and VASP phosphorylation (3 samples). In Stage II, screening and post-intervention flexible

sigmoidoscopy with 8 biopsies obtained from the rectum were performed, with all other parameters and study procedures (including oral MoviPrep) remaining the same. In Stage III, only tap water or PEG enemas were used to cleanse the rectum. Enemas were repeated (up to 3 times) until clear of stool, before screening and post-intervention sigmoidoscopies were performed.

### **Primary Endpoints**

The primary endpoint of the study was to identify a dose of linaclotide that produced a 60% response rate for the pharmacodynamic (PD) endpoint (cGMP level) based on rectal samples obtained at screening and post-intervention. The pharmacological effect of linaclotide (or placebo) was calculated as the arithmetic difference in mean cGMP levels in biopsies from the colonoscopy before and after 7 days of intervention (linaclotide or placebo) in biopsies from the colonoscopy. This represents the change in cGMP stimulated by 7 days of linaclotide in an individual subject. The mean cGMP value was calculated based on 2 biopsies from the rectum assessed at each time point. Each biopsy was analyzed in triplicate using a commercially available enzyme-linked immunosorbent assay (EIA) kit, so that each subject had 6 cGMP values at each time point. PD responses were calculated as difference in mean cGMP levels after 7 days (the Pharmacological Effect) which is  $\geq 0.94$  times the baseline pooled intra-subject standard deviation (SD) of cGMP. The intra-subject standard deviation (SD) was calculated based on the 6 cGMP values at baseline. Cohort size calculations were based on mucosal cGMP data from studies with healthy volunteers (17, 18) and recommendations from a previous Phase 0 study design.(19) This design yielded approximately 89% power to detect a 60% PD response rate at the subject level assuming a 1-sided alpha level of 0.05.(19)

## **Cyclic GMP**

The primary endpoint for all stages was the ability of oral linaclotide to increase cGMP accumulation in colorectal mucosae. The technique for cGMP quantification by immunoassay is well defined.(20) At collection, mucosal biopsies were placed in cryogenic tubes, frozen in liquid nitrogen and archived in a -80°C freezer. For analysis, samples underwent cryopulverization before thawing in 500 µL of pre-cooled 5% trichloroacetic acid (TCA) followed by centrifugation (1,500 rpm, 10 min, 0-4°C). Four hundred (400) µL of the supernatant was extracted with ether to remove TCA and then 250 µL was subjected to cGMP quantification using a validated enzyme-linked immunoassay (Cyclic GMP EIA Kit, Cayman Chemical Company, Ann Arbor, MI). Tissue residues were dissolved in 0.2 N sodium hydroxide at 4°C overnight and protein concentrations determined by BCA protein assay kit (ThermoScientific, Rockford, IL). Cyclic GMP levels were normalized to the protein content from individual samples.

## **Phosphorylation of VASP**

VASP phosphorylation from sites in the colon was quantified by immunoblot analyses of biopsy specimens from normal mucosa employing commercially available antibodies (Phospho-VASP (Ser239) Antibody, Cat: # 3114, Cell Signaling, Danvers, MA). At least two biopsy specimens from each anatomical location were evaluated by two independent immunoblot analyses with quantification by densitometry and normalization to villin (VIL1), and the resulting 4 individual results averaged for comparisons.

## **Ki67**

The impact of linaclotide on cell proliferation index (number of proliferating cells) was quantified employing Ki67 immunohistochemistry (Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Cat. #M7240, DAKO, Carpinteria, CA). At least two biopsy specimens from each anatomical location were evaluated by enumerating Ki67-positive cells in 15 crypts, and the resulting individual crypt cell counts pooled for comparisons.

## **Safety**

To confirm the safety and tolerability of linaclotide and placebo, all participants were monitored for toxicity from the time of their first dose of linaclotide/placebo. CTCAE version 4.0 was used to summarize adverse events.

## **Statistical Analyses**

Frequency tables and percentages summarized baseline and clinical characteristics, treatment data, and adverse event data, overall and by stage for each treatment arm. Descriptive statistics, including mean, standard deviation, median, range, and frequencies (percentages), were used to summarize these data. Fisher's Exact and Wilcoxon Rank-Sum tests were used to test for associations between treatment arms and categorical and continuous data, respectively. All statistical tests were 2-sided and performed using SAS version 9.4 (SAS Institute, Inc.). Associations between treatment arms and secondary endpoints, including Ki67 and VASP phosphorylation, were performed using student's t test, using  $p < 0.05$  as the threshold for significance.

## **Results**

### **Subject Characteristics**

For this study, 46 subjects were screened, with 22 determined to be screen failures (Fig. 1). The 24 subjects enrolled had a mean age of  $47.7 \pm 6.3$  years, 79.2% were male, 45.8% were white, and 54.2% were black (Table 1). Physical exams and laboratory studies revealed no clinically remarkable findings for this cohort of normal healthy volunteers (Supplementary Table 1). Subjects randomized to linaclotide and placebo groups had similar characteristics except for a slightly higher BMI in the linaclotide group (Supplementary Table 1). Six subjects (5 placebo, 1 linaclotide) had polyps >2 mm detected and removed at the pre-intervention colonoscopy (Supplementary Table 2): 3 were tubular adenomas (all received placebo) and 3 were hyperplastic polyps (1 received linaclotide, 2 received placebo).

### **GUCY2C Activation**

#### **Stage I**

In Stage I, 0.87 mg of oral linaclotide for 7 days produced pharmacological responses, increasing cGMP levels in epithelial cells of the cecum (Supplementary Table 3), transverse colon (Supplementary Table 4) and rectum (Supplementary Table 5) in two of three subjects receiving the active agent. Pharmacological responses reflected PD responses in those two subjects in all anatomical sites, including the rectum (Supplementary Tables 3-5, Fig. 2). PD responses in those two subjects were associated with clinical responses of increases in stool frequency and decreases in stool consistency on most days of dosing. In contrast, the subject who received linaclotide but did not have cGMP PD responses also did not experience a change in bowel

movements. Cyclic GMP responses were associated with increases in the phosphorylation of the downstream effector VASP in those subjects, but not in subjects who received placebo or in the one subject that received linaclotide but did not have a PD response (Fig. 3). Similarly, cGMP PD responses to linaclotide were associated with reduced crypt proliferation in all anatomical segments, quantified by Ki67 immunohistochemistry (Fig. 4).

## **Stage II**

PD responses in 2 of 3 actively treated subjects qualified as success, advancing the trial to Stage II (Fig. 1). In this stage of the study, all procedures were identical to Stage I, including an oral bowel preparation, except pre- and post-intervention rectal biopsies were obtained by sigmoidoscopy (assessed only for cGMP levels). As in stage I, 0.87 mg of oral linaclotide for 7 days produced a PD response in 2 subjects, increasing cGMP levels in epithelial cells in the rectum (Fig. 5A) in two of three subjects receiving the active agent. As before, PD responses in those two subjects were associated with an increase in stool frequency and a decrease in stool consistency while the subject who received linaclotide but did not have cGMP PD responses also did not experience a change in bowel movements. Again, PD responses in 2 of 3 actively treated subjects qualified as success, advancing the trial to Stage III (Fig. 1).

## **Stage III**

In this stage of the study, all procedures were identical to stage 2 except subjects received tap water enemas, rather than oral Moviprep, prior to collection of pre- and post-intervention rectal biopsies by sigmoidoscopy and assessment of cGMP levels (Fig. 1). Unlike Stages I and II, 0.87 mg of oral linaclotide for 7 days did not produce a PD response in subjects receiving active

treatment (Fig. 5B). Moreover, no subject in this group experienced a change in bowel movements with linaclotide administration. As these results were unanticipated, we searched the research literature identifying one report suggesting that tap water enemas can disrupt the overlying epithelium sampled by endoscopic biopsy. In that context, changes in cGMP produced by linaclotide only occurred in epithelial cells, since they expressed the GUCY2C receptor. In contrast to tap water, PEG enemas preserve epithelia by endoscopic biopsy.(21) We amended the protocol so Stage III included a cohort that received a PEG enema instead of the tap water enema. However, 0.87 mg of oral linaclotide for 7 days did not produce a PD response in subjects even following the PEG enema with MoviPrep (Fig. 5C). The study was terminated because linaclotide failed to produce a PD response in at least two subjects in this cohort.

## **Safety**

The dose of linaclotide employed here, 0.87 mg, was well tolerated and all subjects completed their full 7 days of dosing without discontinuation or dose reduction. Adverse events were all grade 1 by CTCAE criteria, and all subjects, linaclotide and placebo arms, experienced at least 1 adverse event during the study. Adverse events were similar in both intervention cohorts, except for an increase in bowel frequency and a decrease in consistency, an expected effect of exposure to linaclotide (Supplementary Table 6). Post-intervention endoscopy findings were similar in the linaclotide and placebo groups (Supplementary Table 7).

## **Discussion**

In health, GUCY2C plays a key regulatory role in proliferative and metabolic processes that oppose tumorigenesis. However, the near universal over-expression of GUCY2C in human



colorectal cancers, coupled with the loss of endogenous ligands (guanylin and uroguanylin), highlight a potential targeted prevention strategy for colorectal cancer involving oral replacement therapy. This presumes that during colorectal carcinogenesis, GUCY2C is a dormant tumor-suppressing receptor whose re-engagement by exogenous ligand rescues dysregulated cell growth. In that context, GUCY2C signaling inhibits the cell cycle of normal human intestinal cells and human colon carcinoma cells in vitro and ex vivo.(7, 8, 22) Similarly, GUCY2C signaling reverses the tumorigenic metabolic phenotype in human colon cancer cells.(7, 8) Further, mice on oral uroguanylin demonstrated a decrease in small and large intestine adenoma formation compared to controls. Moreover, hormone loss silencing GUCY2C appears to be required for tumorigenesis since transgenic expression of guanylin, which cannot be suppressed, eliminates carcinogen-induced colorectal tumorigenesis in mice.(14)

Linaclotide, a chemically synthesized 14-amino acid peptide composed of naturally occurring L-amino acids, shares over 60% amino acid identity with guanylin and uroguanylin. This drug is approved by FDA to treat constipation-predominant irritable bowel syndrome (IBS-C) and chronic idiopathic constipation (CIC) under the trade name Linzess™.(23, 24) Linaclotide enhances bowel function by activating GUCY2C and inducing fluid and electrolyte secretion in the small intestine, improving frequency and stool consistency. Generally, linaclotide is well tolerated, with side effects primarily reflecting on-target activity of GUCY2C mediating fluid and electrolyte secretion underlying diarrhea.(23, 24) The robust safety of this agent is underscored by the negligible bioavailability of orally administered linaclotide.(16, 25) While treatment of chronic constipation syndromes usually involves daily oral linaclotide doses of 0.145 or 0.290 mg,(23, 24) daily doses up to 1 mg are safe. Here, a dose of 0.87 mg was selected because the

primary goal was to determine whether linaclotide activated GUCY2C signaling in the distal rectum, a site that is exposed only to ~1-3% of the oral dose likely reflecting proteolysis.(16, 25)

A maximum oral dose of linaclotide (0.87 mg) administered for 7 days increased cGMP, associated with changes in VASP phosphorylation and Ki67 staining, in biopsy specimens from the cecum, transverse colon, and rectum, obtained by colonoscopy following bowel preparation by oral MoviPrep administration in some healthy volunteers (Stage I). Similarly, 0.87 mg of oral linaclotide for 7 days increased cGMP accumulation in epithelial cells of the rectum recovered by sigmoidoscopy following oral MoviPrep bowel preparation in some healthy volunteers (Stage II). In each of these cohorts, changes in cGMP were associated with changes in frequency and/or stool consistency induced by linaclotide. Conversely, subjects administered linaclotide that did not experience changes in bowel movements did not exhibit changes in mucosal cGMP. Importantly, linaclotide failed to increase rectal mucosa cGMP in subjects in which rectal stool was cleared by enema (Stage III), in the absence of oral MoviPrep.

We can only speculate about mechanisms that prevented linaclotide from activating rectal cGMP production in Stage III. First, it is noteworthy that no subject in Stage III experienced changes in bowel movements with linaclotide. Previous clinical studies revealed that ~20-30% of patients did not experience changes in bowel movements when administered linaclotide.(26) This variability in response could reflect genetic factors affecting the pharmacodynamics of linaclotide in epithelial cells in some individuals. In that context, GUCY2C expression in normal epithelia varies about 2 orders of magnitude in the population.(27) Further, mutations that alter GUCY2C activity have been described.(28-31) Alternatively, these differences may relate to pharmacokinetic polymorphisms, with differences in metabolic clearance of the peptide in

the intestine limiting the availability of active drug in some patients. Moreover, the contribution of environmental factors extrinsic to epithelial cells, for example variations in the microbiome, might contribute to the variability in individual responses to linaclotide. These possibilities remain to be explored.

Additionally, the inactivity of linaclotide in Stage III could reflect the bowel preparation employed on the last day of dosing—a laxative preparation effect. In Stages I and II, subjects received an oral dose of MoviPrep to clear stool from the colorectum prior to the last dose of linaclotide and endoscopy. In both of these first two stages, linaclotide elevated cGMP in rectal mucosa in some subjects. However, in the absence of oral MoviPrep before the last dose of drug, linaclotide was ineffective in elevating cGMP in rectal mucosa. It is tempting to speculate that changes in cGMP, and downstream effectors, in mucosa from the colorectum observed in Stage I and II reflected only the last dose of linaclotide. Indeed, GUCY2C interactions with ligands occur with rapid on-off kinetics, and cGMP production reflects receptor occupancy, without persistence following ligand dissociation. In that context, oral MoviPrep may have cleared stool and increased intestinal transit, delivering a greater quantity of linaclotide more rapidly to the colorectum in Stages I and II. Alternatively, the presence of stool throughout the colorectum on day 7 may have prevented delivery of active linaclotide to the colorectal mucosa, possibly reflecting the established surface-active characteristics of GUCY2C ligands and the resulting immobilization of linaclotide in the solid phase of the intestinal contents. Again, these possibilities remain to be explored.

These observations suggest concrete steps for advancing GUCY2C as a target for colorectal cancer chemoprevention by oral hormone replacement therapy. For example, future studies

should identify subjects who are biological responders to linaclotide, to avoid enrolling subjects who are insensitive to this agent because of pharmacokinetic or pharmacodynamic differences. Further, it would be useful to understand the molecular mechanisms underlying insensitivity to GUCY2C ligands in the population to better generalize the ultimate chemoprevention strategy to the greatest number of patients. Moreover, it will be important to test sustained release formulations of linaclotide that are targeted to the colorectum, to maximize pharmacodynamic effects of GUCY2C activation and downstream signaling mediating chemoprevention.

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## References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010;60:277-300.
2. Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev.* 2000;52:375-414.
3. Field M. Mechanisms of action of cholera and Escherichia coli enterotoxins. *Am J Clin Nutr.* 1979;32:189-96.
4. Guerrant RL, Hughes JM, Chang B, Robertson DC, Murad F. Activation of intestinal guanylate cyclase by heat-stable enterotoxin of Escherichia coli: studies of tissue specificity, potential receptors, and intermediates. *J Infect Dis.* 1980;142:220-8.
5. Guarino A, Cohen M, Thompson M, Dharmasathaphorn K, Giannella R. T84 cell receptor binding and guanyl cyclase activation by Escherichia coli heat-stable toxin. *Am J Physiol.* 1987;253:G775-80.
6. Li P, Lin JE, Snook AE, Gibbons AV, Zuzga DS, Schulz S, et al. Colorectal cancer is a paracrine deficiency syndrome amenable to oral hormone replacement therapy. *Clin Transl Sci.* 2008;1:163-7.
7. Li P, Lin JE, Chervoneva I, Schulz S, Waldman SA, Pitari GM. Homeostatic control of the crypt-villus axis by the bacterial enterotoxin receptor guanylyl cyclase C restricts the proliferating compartment in intestine. *Am J Pathol.* 2007;171:1847-58.
8. Lin JE, Li P, Snook AE, Schulz S, Dasgupta A, Hyslop TM, et al. The hormone receptor GUCY2C suppresses intestinal tumor formation by inhibiting AKT signaling. *Gastroenterology.* 2010;138:241-54.

9. Steinbrecher KA, Tuohy TM, Heppner Goss K, Scott MC, Witte DP, Groden J, et al. Expression of guanylin is downregulated in mouse and human intestinal adenomas. *Biochem Biophys Res Commun.* 2000;273:225-30.
10. Notterman DA, Alon U, Sierk AJ, Levine AJ. Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. *Cancer Res.* 2001;61:3124-30.
11. Birkenkamp-Demtroder K, Christensen LL, Olesen SH, Frederiksen CM, Laiho P, Aaltonen LA, et al. Gene expression in colorectal cancer. *Cancer Res.* 2002;62:4352-63.
12. Waldman SA, Cagir B, Rakinic J, Fry RD, Goldstein SD, Isenberg G, et al. Use of guanylyl cyclase C for detecting micrometastases in lymph nodes of patients with colon cancer. *Dis Colon Rectum.* 1998;41:310-5.
13. Birbe R, Palazzo JP, Walters R, Weinberg D, Schulz S, Waldman SA. Guanylyl cyclase C is a marker of intestinal metaplasia, dysplasia, and adenocarcinoma of the gastrointestinal tract. *Hum Pathol.* 2005;36:170-9.
14. Lin JE, Colon-Gonzalez F, Blomain E, Kim GW, Aing A, Stoecker B, et al. Obesity-Induced Colorectal Cancer Is Driven by Caloric Silencing of the Guanylin-GUCY2C Paracrine Signaling Axis. *Cancer Res.* 2016;76:339-46.
15. Shailubhai K, Yu HH, Karunanandaa K, Wang JY, Eber SL, Wang Y, et al. Uroguanylin treatment suppresses polyp formation in the *Apc(Min/+)* mouse and induces apoptosis in human colon adenocarcinoma cells via cyclic GMP. *Cancer Res.* 2000;60:5151-7.
16. Busby RW, Kessler MM, Bartolini WP, Bryant AP, Hannig G, Higgins CS, et al. Pharmacologic properties, metabolism, and disposition of linaclotide, a novel therapeutic

peptide approved for the treatment of irritable bowel syndrome with constipation and chronic idiopathic constipation. *J Pharmacol Exp Ther.* 2013;344:196-206.

17. Corazza GR, Ciccarelli R, Caciagli F, Gasbarrini G. Cyclic AMP and cyclic GMP levels in human colonic mucosa before and during chenodeoxycholic acid therapy. *Gut.* 1979;20:489-92.

18. Kuhn M, Adermann K, Jahne J, Forssmann WG, Rechkemmer G. Segmental differences in the effects of guanylin and Escherichia coli heat-stable enterotoxin on Cl<sup>-</sup> secretion in human gut. *J Physiol.* 1994;479 ( Pt 3):433-40.

19. Murgo AJ, Kummar S, Rubinstein L, Gutierrez M, Collins J, Kinders R, et al. Designing phase 0 cancer clinical trials. *Clin Cancer Res.* 2008;14:3675-82.

20. Steiner AL, Pagliara AS, Chase LR, Kipnis DM. Radioimmunoassay for cyclic nucleotides. II. Adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in mammalian tissues and body fluids. *J Biol Chem.* 1972;247:1114-20.

21. Schmelzer M, Schiller LR, Meyer R, Rugari SM, Case P. Safety and effectiveness of large-volume enema solutions. *Appl Nurs Res.* 2004;17:265-74.

22. Pitari GM, Di Guglielmo MD, Park J, Schulz S, Waldman SA. Guanylyl cyclase C agonists regulate progression through the cell cycle of human colon carcinoma cells. *Proc Natl Acad Sci U S A.* 2001;98:7846-51.

23. Chey WD, Lembo AJ, Lavins BJ, Shiff SJ, Kurtz CB, Currie MG, et al. Linaclotide for irritable bowel syndrome with constipation: a 26-week, randomized, double-blind, placebo-controlled trial to evaluate efficacy and safety. *Am J Gastroenterol.* 2012;107:1702-12.

24. Lembo AJ, Schneier HA, Shiff SJ, Kurtz CB, MacDougall JE, Jia XD, et al. Two randomized trials of linaclotide for chronic constipation. *N Engl J Med.* 2011;365:527-36.



25. Busby RW, Bryant AP, Bartolini WP, Cordero EA, Hannig G, Kessler MM, et al. Linaclotide, through activation of guanylate cyclase C, acts locally in the gastrointestinal tract to elicit enhanced intestinal secretion and transit. *Eur J Pharmacol.* 2010;649:328-35.
26. Bharucha AE, Waldman SA. Taking a lesson from microbial diarrheagenesis in the management of chronic constipation. *Gastroenterology.* 2010;138:813-7.
27. Waldman SA, Hyslop T, Schulz S, Barkun A, Nielsen K, Haaf J, et al. Association of GUCY2C expression in lymph nodes with time to recurrence and disease-free survival in pN0 colorectal cancer. *Jama.* 2009;301:745-52.
28. Fiskerstrand T, Arshad N, Haukanes BI, Tronstad RR, Pham KD, Johansson S, et al. Familial diarrhea syndrome caused by an activating GUCY2C mutation. *N Engl J Med.* 2012;366:1586-95.
29. Muller T, Rasool I, Heinz-Erian P, Mildemberger E, Hulstrunk C, Muller A, et al. Congenital secretory diarrhoea caused by activating germline mutations in GUCY2C. *Gut.* 2016;65:1306-13.
30. Romi H, Cohen I, Landau D, Alkrinawi S, Yerushalmi B, Hershkovitz R, et al. Meconium ileus caused by mutations in GUCY2C, encoding the CFTR-activating guanylate cyclase 2C. *Am J Hum Genet.* 2012;90:893-9.
31. Smith A, Bulman DE, Goldsmith C, Bareke E, Majewski J, Boycott KM, et al. Meconium ileus in a Lebanese family secondary to mutations in the GUCY2C gene. *Eur J Hum Genet.* 2015;23:990-2.

**Table 1. Baseline Demographics**

	Linacotide (N=12)	Placebo (N=12)	Total (N=24)	p value
<b>Gender</b>				0.32 <sup>1</sup>
Female	4 (33.3%)	1 (8.3%)	5 (20.8%)	
Male	8 (66.7%)	11 (91.7%)	19 (79.2%)	
<b>Age</b>				1.00 <sup>2</sup>
N	12	12	24	
Mean (SD)	47.9 (5.8)	47.5 (7.0)	47.7 (6.3)	
Median	48.0	49.5	48.0	
Q1, Q3	45.0, 51.5	44.0, 52.0	45.0, 51.5	
Range	(35.0-58.0)	(35.0-57.0)	(35.0-58.0)	
<b>Race</b>				1.00 <sup>1</sup>
White	6 (50.0%)	5 (41.7%)	11 (45.8%)	
Black or African American	6 (50.0%)	7 (58.3%)	13 (54.2%)	
<b>Ethnicity</b>				0.48 <sup>1</sup>
Hispanic or Latino	0 (0.0%)	2 (16.7%)	2 (8.3%)	
Non-Hispanic	12 (100.0%)	10 (83.3%)	22 (91.7%)	
<sup>1</sup> Fisher Exact <sup>2</sup> Wilcoxon				

## Figure Legends

**Figure 1. CONSORT flow diagram of subject progress through the phases of the clinical trial.**

**Figure 2. Cyclic GMP pharmacodynamic response to linaclotide or placebo in healthy volunteers in Stage I.** Cyclic GMP pharmacodynamic response was calculated as described in Methods. C, cecum; TC, transverse colon, R, rectum.

**Figure 3. VASP phosphorylation in mucosal biopsies from healthy subjects treated with linaclotide or placebo in Stage I.** Phosphorylated VASP was quantified by densitometry following immunoblot analysis of biopsies from the cecum, transverse colon, and rectum. The amount of phosphorylated VASP was normalized to the epithelial marker villin. For each intestinal segment, the ratio of normalized phosphorylated villin on day 1 (pre-dose) and 7 (post-dose) were calculated. \*,  $p < 0.05$ .

**Figure 4. Cell proliferation in crypts in mucosal biopsies from healthy subjects treated with linaclotide or placebo in Stage I.** Proliferation was quantified by enumerating cells expressing Ki67 by immunofluorescence. Ki67 was enumerated in 10-20 crypts in each biopsy and means were calculated. For each intestinal segment, the ratio of mean Ki67 expression on day 1 (pre-dose) and 7 (post-dose) were calculated. C, cecum; TC, transverse colon, R, rectum. \*\*\*,  $p < 0.001$ .

**Figure 5. Cyclic GMP pharmacodynamic response to linaclotide or placebo in healthy volunteers in Stage II and III.** Cyclic GMP pharmacodynamic response was calculated as described in Methods in rectal biopsies of healthy subjects in (A) Stage II, (B) Stage III following tap water enemas, and (C) Stage III following PEG enemas.