Bioactivity of Oral Linaclotide in Human Colorectum for Cancer Chemoprevention


Abstract

Guanylate cyclase C (GUCY2C) is a tumor-suppressing receptor silenced by loss of expression of its luminal core 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 mg 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.

Introduction

Colorectal cancer is the fourth 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 (acetylsalicylic acid) and other 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 acetylsalicylic acid 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, 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 coopted by bacteria that secrete heat-stable enterotoxins, 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 overexpress GUCY2C compared with normal adjacent mucosa.
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 and 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 preclinical 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 chemoprevention-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.) 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 approximately 1% to 3% of orally administered linaclotide or its active metabolite is recovered in stool (16). This 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 7 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 sigmoidoscopic biopsy following oral bowel preparation. Stage III explored the ability of that same dose to activate rectal GUCY2C (the most distant site for chemoprevention) by sigmoidoscopic 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 sigmoidoscopic sampling following rectal preparation by tap water or PEG enema. Stage III was designed to determine whether 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 sigmoidoscopic 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 (Rochester, MN), Thomas Jefferson University (Philadelphia, PA), and Fox Chase Cancer Center and registered on ClinicalTrials.gov (NCT01950403). The study population included healthy volunteers 18 to 65 years old, without personal or first-degree family history of colorectal cancer, inflammatory bowel disease, or other recent (≤3 months prior to day 0)
or ongoing diseases producing acute or chronic diarrhea. In stage I, subjects received oral bowel preparation with 100 g 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 hours 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 postintervention colonoscopies; 8 from each of the 3 anatomic locations, including cecum, transverse colon, and rectum. Three samples from each anatomic site were flattened immediately and fixed in prechilled paraformaldehyde (4%) overnight followed by standard tissue processing for analysis of Ki67. The remaining five samples from each anatomic location were flash frozen in liquid nitrogen and stored at −80°C for analysis of cGMP levels (two samples) and VASP phosphorylation (three samples). In stage II, screening and postintervention flexible sigmoidoscopy with 8 biopsies obtained from the rectum were performed along 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 postintervention 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 endpoint (cGMP level) based on rectal samples obtained at screening and postintervention. The pharmacologic 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 on the basis of two biopsies from the rectum assessed at each time point. Each biopsy was analyzed in triplicate using a commercially available ELISA Kit, so that each subject had 6 cGMP values at each time point. Pharmacodynamic responses were calculated as difference in mean cGMP levels after 7 days (the pharmacologic effect), which is ≥0.94 times the baseline pooled intrasubject SD of cGMP. The intrasubject SD was calculated on the basis of 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% pharmacodynamic response rate at the subject level assuming a one-sided alpha level of 0.05 (19).

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 Technology). At least two biopsy specimens from each anatomic location were evaluated by two independent immunoblot analyses with quantification by densitometry and normalization to villin (VIL1), and the resulting four individual results averaged for comparisons.

Ki67
The impact of linaclotide on cell proliferation index (number of proliferating cells) was quantified employing Ki67 IHC (Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Cat. #M7240, DAKO). At least two biopsy specimens from each anatomic location were evaluated by enumerating Ki67-positive cells in 15 crypts, and the resulting individual crypt cell counts were 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, SD, median, range, and frequencies (percentages), were used to summarize these data. Fisher 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 two-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 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 ± 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 S1). Subjects randomized to linaclotide and placebo groups had similar characteristics except for a slightly higher BMI in the linaclotide group (Supplementary Table S1). Six subjects (5 placebo, 1 linaclotide) had polyps >2 mm detected and removed at the preintervention colonoscopy (Supplementary Table S2): 3 were tubular adenomas (all received placebo) and 3 were hyperplastic polyps (1 received linaclotide, 2 received placebo).

Table 1. Baseline demographics

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<td>12</td>
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*Fisher exact.
*Wilcoxon.

GUCY2C activation

Stage I. In stage I, 0.87 mg of oral linaclotide for 7 days produced pharmacologic responses, increasing cGMP levels in epithelial cells of the cecum (Supplementary Table S3), transverse colon (Supplementary Table S4) and rectum (Supplementary Table S5) in 2 of 3 subjects receiving the active agent. Pharmacologic responses reflected pharmacodynamic responses in those 2 subjects in all anatomic sites, including the rectum (Supplementary Tables S3–S5, Fig. 2). Pharmacodynamic responses in those 2 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 pharmacodynamic 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 pharmacodynamic response (Fig. 3). Similarly, cGMP pharmacodynamic responses to linaclotide were associated with reduced crypt proliferation in all anatomic segments, quantified by Ki67 IHC (Fig. 4).

Stage II. Pharmacodynamic 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 postintervention 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 pharmacodynamic response in 2 subjects, increasing cGMP levels in epithelial cells in the rectum (Fig. 5A) in 2 of 3 subjects receiving the active agent. As before, pharmacodynamic responses in those 2 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 pharmacodynamic responses also did not experience a change in bowel movements. Again, pharmacodynamic 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 II except subjects received tap water enemas, rather than oral MoviPrep, prior to collection of pre- and postintervention 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 pharmacodynamic 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
A 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, as they expressed the GUCY2C receptor. In contrast to tap water, PEG enemas preserve epithelia by endoscopic biopsy. 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 pharmacodynamic response in subjects even following the PEG enema with MoviPrep. The study was terminated because linaclotide failed to produce a pharmacodynamic response in at least 2 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 S6). Postintervention endoscopy findings were similar in the linaclotide and placebo groups (Supplementary Table S7).

**Discussion**

In health, GUCY2C plays a key regulatory role in proliferative and metabolic processes that oppose tumorigenesis. However, the near universal overexpression of GUCY2C in human colorectal cancers, coupled with the loss of endogenous ligands (guanylin and uroguanylin), highlights 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 reengagement 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. Similarly, GUCY2C signaling reverses the tumorigenic metabolic phenotype in human colon cancer cells.

Moreover, mice on oral uroguanylin demonstrated a decrease in small and large intestine adenoma formation compared with controls. Moreover, hormone loss silencing GUCY2C appears to be required for tumorigenesis as transgenic expression of guanylin, which cannot be suppressed, eliminates carcinogen-induced colorectal tumorigenesis in mice.

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 and chronic idiopathic constipation 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). Although 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 approximately 1% to 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 approximately 20% to 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 two orders of magnitude in the population (27). Furthermore, mutations that alter GUCY2C activity have been described previously (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.

In addition, the inactivity of linaclotide in stage III could reflect the bowel preparation employed on the last day of dosing, a
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. Furthermore, 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.S. Weinberg, J.E. Lin, N.R. Foster, G. Della’Zanna, A. Umar, P.J. Limburg

Figure 5.
CGMP pharmacodynamic response to linaclotide or placebo in healthy volunteers in stage II and III. A–C, cGMP pharmacodynamic response was calculated as described in Materials and Methods in rectal biopsies of healthy subjects in stage II (A), stage III following tap water enemas (B), and stage III following PEG enemas (C).

Linaclotide Bioactivity in Human Colorectum

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.S. Weinberg, J.E. Lin, N.R. Foster, D. Seisler, P.J. Limburg

Writing, review, and/or revision of the manuscript: D.S. Weinberg, J.E. Lin, N.R. Foster, G. Della’Zanna, A. Umar, D. Seisler, D.M. Kastenberg, P.J. Limburg

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.S. Weinberg, N.R. Foster, G. Della’Zanna

Study supervision: D.S. Weinberg, W.K. Kraft, D.M. Kastenberg, P.J. Limburg, S.A. Waldman

Other (study principal investigator): D.S. Weinberg

Other (performed required procedures): L.C. Katz

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