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Linaclotide, through activation of guanylate cyclase C, acts locally in the gastrointestinal tract to elicit enhanced intestinal secretion and transit

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ABSTRACT

Linaclotide is a first-in-class, orally administered 14-amino acid peptide that is in development for the treatment of irritable bowel syndrome with constipation and chronic constipation. We have characterized the solution structure of linaclotide, the in vitro binding and agonist activity to guanylate cyclase C receptors, the stability of linaclotide under conditions mimicking the gastric environment, oral bioavailability, and the pharmacodynamic effects in rat models of gastrointestinal transit and intestinal secretion. Nuclear magnetic resonance spectroscopy analysis determined that the molecular structure of linaclotide is stabilized by three intramolecular disulfide bridges. Linaclotide exhibited high affinity and pH-independent binding (K_i: 1.23–1.64 nM) to guanylate cyclase C receptors on human colon carcinoma T84 cells and concomitantly, linaclotide binding resulted in a significant, concentration-dependent accumulation of intracellular cyclic guanosine-3′, 5′-monophosphate (cGMP) (EC_{50}:99 nM). Linaclotide was stable after 3 h incubation in simulated gastric fluid (pH 1) and similarly, was completely resistant to hydrolysis by pepsin. Pharmacokinetic analysis of linaclotide showed very low oral bioavailability (0.1%). Orally administered linaclotide elicited a significant, dose-dependent increase in gastrointestinal transit rates in rats at doses of ≥5 μg/kg. Exposure of surgically ligated small intestinal loops to linaclotide induced a significant increase in fluid secretion, accompanied by a significant increase in intraluminal cGMP levels. These results suggest that the guanylate cyclase C agonist linaclotide elicits potent pharmacological responses locally in the gastrointestinal tract, and that orally administered guanylate cyclase C agonists may be capable of improving bowel habits in patients suffering from irritable bowel syndrome with constipation and chronic constipation.

1. Introduction

Linaclotide is a first-in-class, orally administered 14 amino acid peptide of the guanylin peptide family, a family of cyclic guanosine-3′, 5′-monophosphate (cGMP) regulating peptide hormones that also includes the heat-stable microbial ST peptides (Currie et al., 1992; Forte, 2004; Hamra et al., 1993). Guanylin and uroguanylin are primarily expressed along the longitudinal axis of the gastrointestinal tract and released into the intestinal lumen, but their expression pattern and intestinal sites of activity are distinctively different (Fan et al., 1996; Hamra et al., 1993). While uroguanylin exerts its activity primarily in the acidic microenvironment of the proximal duodenum, guanylin is more active in intestinal regions with a more neutral pH microenvironment. Guanylate cyclase C, which is predominantly expressed on the luminal surface of intestinal epithelial cells, is an important regulator of intestinal function and is the molecular target of guanylin and uroguanylin (Schulz et al., 1990). Stimulation of guanylate cyclase C receptors by these peptides leads to increased intracellular levels of the second messenger cGMP. Cyclic GMP is involved in the regulation of a broad range of physiological processes, including the control of intestinal fluid homeostasis (Schlossman et al., 2005; Seidler et al., 1997). Studies in mice genetically deficient in the guanylate cyclase C gene have confirmed the critical role of this receptor in the regulation of intestinal fluid homeostasis (Mann et al., 1997; Schulz et al., 1997). Thus, the guanylate cyclase C receptor appears to be a novel therapeutic target for the treatment of functional gastrointestinal disorders such as irritable bowel syndrome with constipation and chronic constipation.

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Irritable bowel syndrome is a highly prevalent, chronic condition that affects 20 to 64 million individuals in the US (Borum, 2001). Since there is no known structural or biochemical etiology, irritable bowel syndrome is characterized as a functional gastrointestinal disorder (Ringel et al., 2001). Principal symptoms of irritable bowel syndrome include abdominal pain or discomfort that is associated with a change in stool frequency or stool form (Drossman et al., 1999; Drossman, 2006). Chronic constipation, a functional gastrointestinal disorder affecting approximately 15% of the US population, also results in patients' reduced quality of life both physically and psychologically (McCallum et al., 2009). Principal symptoms of chronic constipation include infrequent stools, straining, a feeling of incomplete evacuation, rectal and perianal fullness, abdominal discomfort and bloating (McCallum et al., 2009). Despite significant efforts over the past two decades to develop effective drugs for the treatment of these functional gastrointestinal disorders, therapeutic options are limited. Hence, there is a continued medical need for more effective and safer therapeutic agents.

In this study, we report the solution structure of linaclotide and the in vitro binding and agonist activity to guanylate cyclase C receptors. Furthermore, we have determined the stability of linaclotide under conditions mimicking the gastric environment, the rat oral bioavailability of linaclotide, and whether the pharmacodynamic effects elicited locally in the gastrointestinal tract in rat models of gastrointestinal transit and intestinal secretion are linked to the activation of guanylate cyclase C receptors.

2. Materials and methods

2.1. Animals

Male and female CD rats were obtained from Charles River Laboratories (Wilmington, MA). The animals were housed in a temperature (21 °C ± 1 °C) and relative humidity (35% ± 5%) controlled room with 12-h light/dark cycle, with ad libitum access to filtered tap water and standard irradiated pelleted laboratory chow. The rats were provided with at least a 3-day acclimatization period prior to the start of any experiment. At the end of the studies, rats were euthanized by CO_2 asphyxiation. All animal studies were approved by the Ironwood Pharmaceuticals Institutional Animal Care and Use Committee.

2.2. Test articles

All lots of linaclotide were synthesized at Polypeptide Laboratories (Torrance, CA). [^{125}I]-*Escherichia coli* heat-stable enterotoxin (STa) was radiolabeled at Perkin-Elmer Life and Analytical Sciences (Billerica, MA). Guanylin, uroguanylin, STa, pepsin and oxidized insulin B chain were purchased from Sigma (St. Louis, MS).

2.3. Solution structure of linaclotide

For nuclear magnetic resonance (NMR) spectroscopy, freeze dried linaclotide was dissolved to a final concentration of 5 mM in either 95% H_2O/5% D_2O or 100% D_2O and the pH adjusted to 5.0 with NaOH or NaOD. All NMR spectra were acquired at 280 K on a (500 MHz) NMR spectrometer. Total Correlation Spectroscopy (TOCSY) spectra were obtained from linaclotide in H_2O (90 ms mixing time) and D_2O (70 ms mixing time). Nuclear Overhauser Effect Spectroscopy (NOESY) spectra were acquired from linaclotide solutions in H_2O and D_2O (200 and 500 ms mixing time). ^{13}C-Heteronuclear Single Quantum Coherence (HSQC) spectra were taken in H_2O. Distance restraints for structure calculation were taken from the NOESY spectrum and calculated using the XEASY, NMRPIPE and XPLOR programs.

2.4. Preparation of intestinal mucosa cells and cell culture

The human colon carcinoma cell line T84 was obtained from ATCC (Manassas, VA). Rat intestinal mucosa cells were prepared from the duodenal and jejunal regions of the small intestine. The mucosa was extruded following a modified protocol (Kessler et al., 1970). Briefly, the intestine was placed in a 100 mm plastic dish containing 10 ml phosphate-buffered saline (PBS), pH 7.4 and a P1000 pipet tip was pressed against the intestine several times to extrude the mucosa. The mucosa was collected by centrifugation ($1000 \times g$), washed twice in 20 ml PBS and resuspended in 5 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20 mM HEPES (*N*-(2-hydroxymethyl) piperazine-*N*′-(2-ethanesulfonic acid)) pH 7.0.

2.5. Competitive radioligand binding on T84 cells

Intact human T84 cells were used for competitive radioligand binding experiments. Monolayers of T84 cells were cultured in T-150 plastic flasks at 60–70% confluency, collected by gentle scraping and centrifugation, and washed twice with PBS. [^{125}I]-STa (2200 Ci/mmol) was radiolabeled and purified as described by Thompson et al. (1985). Of the two monoiodinated forms of STa generated, the one labeled at the fourth tyrosine was isolated, purified and used as the tracer in this study (Thompson et al., 1985). The binding reactions were carried out in 1.5 ml microfuge tubes containing 0.24 ml of DMEM/20 mM HEPES pH 7.0/0.5% BSA containing: 2.5×10^5 T84 cells (0.25 mg protein), 200,000 cpm [^{125}I]-STa (41 fmol, 170 pM), and 0.01 to 1000 nM competitor. Binding assays at pH 5 were done in DMEM/20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES). Binding assays at pH 8 were performed in DMEM/20 mM HEPES/50 mM sodium bicarbonate. For the binding reactions at pH 5 and pH 8, competitor concentrations ranged between 0.1 and 3000 nM. Control reactions contained no competitor (B_0) or no cells. After incubation at 37 °C for 1 h, the reactions were applied to Whatman GF/B or GF/C glass-fiber filters (pretreated with 1% polyvinylpyrrolidone) by vacuum filtration. The filters were then rinsed with ice-cold PBS buffer and the trapped radioligand measured in a scintillation counter. Competitive radioligand binding curves were generated using GraphPad Prism (GraphPad Sofware, San Diego, CA). Nonlinear regression analysis of the binding data was used to calculate the concentration of competitor that resulted in 50% radioligand bound (IC_{50}). The apparent dissociation equilibrium constant (K_i) for each competitor was obtained from the IC_{50} values and the previously reported estimate of the dissociation constant for the radioligand, $K_d \cong 15$ nM (Hamra et al., 1997) using the method of Cheng and Prusoff (1973). Because the radioligand concentration of 170 pM used in these assays was very small compared to its dissociation constant, the calculated IC_{50} and the K_i values are in effect identical.

2.6. Competitive radioligand binding on rat intestinal mucosa cells

Binding reactions on intestinal mucosa cells were carried out in 1.5 ml microfuge tubes containing 0.2 ml binding buffer (DMEM/20 mM HEPES pH 7.0/0.25% BSA), rat intestinal mucosa cells (0.9 mg protein), 50,000 cpm [^{125}I]-STa (10 fmol, 42 pM), and 0.01 to 1000 nM linaclotide. Control reactions contained no competitor (B_0) or no cells. The reactions were incubated for 60 min at room temperature to reduce protease activity and then loaded onto Whatman GF/B glass–fiber filters by vacuum filtration. The filters were rinsed with ice-cold PBS and bound [^{125}I]-STa was measured by scintillation counting.

2.7. Cyclic GMP accumulation in T84 cells

Monolayers of human colonic T84 cells were cultured in 24-well dishes to approximately 85% confluency in DMEM. The cells in each well were washed twice with DMEM, and 450 µl of DMEM containing 1 mM isobutylmethylxanthine (IBMX) were then added. Plates were

incubated for 10 min in a 37 °C/5% CO_2 incubator. Fifty μl of the appropriate 10-fold concentration of synthetic peptide were added to the appropriate wells (n = 4/concentration). After 30 min of peptide exposure at 37 °C /5% CO_2, the media from each well was aspirated and the cells were lysed by addition of 500 μl of ice-cold 0.1 M HCl per well. Cell lysates were added to a 96-deep well polypropylene plate and dried using vacuum evaporation. Samples were resuspended in 500 μl of phosphate buffer (Cayman enzyme immunoassay (EIA) cyclic GMP kit) and stored at −20 °C. The concentration of cGMP was determined using a competitive enzyme immunoassay kit as indicated by the manufacturer (Cayman Chemicals, Ann Arbor, MI). EC_{50} values were calculated from concentration–response curves generated with GraphPad Prism (GraphPad Software, San Diego, CA).

2.8. Incubation of linaclotide in simulated gastric fluid

Linaclotide (153 μg/ml) was prepared in 500 μl simulated gastric fluid (0.2 NaCl (w/v), 0.7% HCl (v/v), pH 1) and incubated for 0, 1 or 3 h at 37 °C. A linaclotide reference control in distilled water was incubated under the same conditions. The concentration of linaclotide at 0, 1 or 3 h was quantified by LC/MS using a standard curve of linaclotide (0.625, 1.25, 2.50, 5.0, and 10.0 μM) prepared in distilled water.

2.9. Incubation of linaclotide with pepsin

Pepsin (100 U/ml) samples were prepared in 500 μl pepsin reaction buffer (100 mM HCl–KCl buffer, pH 2). Fifty μl of linaclotide (0.1 mg/ml) was added and the reactions were incubated for 0, 1 or 3 h at 37 °C. Parallel reactions were incubated with 50 μl insulin B chain, oxidized (0.1 mg/ml) under the same conditions. Reactions were quenched with 500 μl of 1 M ammonium acetate and were stored at 4 °C until analysis by LC/MS. For quantification, standards of linaclotide and insulin B chain, oxidized (0.625, 1.25, 2.50, 5.0, and 10 μg/ml each) were prepared in 25 mM Tris–HCl, 500 mM NaCl, pH 7.5.

2.10. Oral bioavailability of linaclotide in rats

To determine oral bioavailability, 4 groups (n = 3) of rats received linaclotide (10 mg/kg) intravenously (i.v.), while 4 groups (n = 3) received linaclotide (10 mg/kg) by gavage (p.o.). Blood was drawn from the retro-orbital sinus, collected in plasma tubes containing the potassium salt of ethylenediaminetetraacetic acid (K_2EDTA), centrifuged at 13,000×*g* for 3 min and plasma was stored at −80 °C until sample preparation and analysis by LC/MS. The concentration of linaclotide was determined from peak response relative to a set of standards prepared in rat plasma (limit of detection: 0.2 ng/ml). Data were collected using Waters MassLynx version 4.00 software. Linaclotide plasma concentrations were plotted as a function of time using GraphPad Prism 4.0 software, which was also used to calculate the area under the linaclotide oral and i.v. curves. If no analyte was detected, the concentration was arbitrarily set to zero. Oral bioavailability (F) was calculated using the equation: $F = (AUC_{p.o.(0–6\ h)})/(AUC_{i.v.(0–6\ h)})$ given that the intravenous and oral dose of linaclotide were identical.

2.11. Small intestinal loop ligation and secretion studies

The effects of linaclotide on secretion and on cGMP levels were studied by injecting linaclotide directly into isolated duodenal, jejunal and ileal loops in female CD rats (n = 10/group), which had been surgically ligated following a modified method by London et al. (1997). Loops were 1 to 3 cm in length and were injected with 200 μl of either linaclotide (5 μg) or vehicle (Krebs Ringer, 10 mM glucose, HEPES buffer). Animals were allowed a recovery time of 90 min following surgery after which they were euthanized and the loops excised. The lengths and weight of the intestinal loops were recorded both before and after emptying of the fluid contents. A weight to length ratio (W/L) for each loop was calculated and expressed as a percentage of the control value, a commonly used surrogate to measure intestinal secretion (Thiagarajah et al., 2004).

2.12. Purification and extraction of cGMP from ligated loop fluid

Samples of fluid obtained from rat ligated intestinal loops (n = 10/group) were collected in 15 ml Falcon tubes and immediately placed in 300 μl of 6% ice-cold trichloroacetic acid (TCA) for every 100 μl of sample, and stored at −20°C until assayed. After samples were thawed on ice, they were homogenized for 30 s using an Omni International GLH type homogenizer. The precipitate was removed by centrifugation at 1500×*g* for 10 min. The supernatant solution was then transferred into a clean 15 ml Falcon tube. TCA was extracted from the sample using water-saturated ether. Five volumes of ether to one volume of supernatant was added to the sample and then mixed for 10 s and the organic and aqueous layers were allowed to separate. The top layer was carefully removed and discarded and the extraction repeated three more times. Any residual ether was removed from the aqueous layer by heating the sample to 70°C for 5 min in a water bath. Samples were stored at −20 °C until ready for cGMP quantification. Cyclic GMP levels were determined using a competitive enzyme immunoassay kit as described in Section 2.7.

2.13. Gastrointestinal transit in rats — acute

A commonly accepted model to measure gastrointestinal transit in the upper gastrointestinal tract of rats was used, since measurements of colonic transit in rats have been proven difficult due to the fluid reservoir function of the cecum during periods of intestinal hypersecretion (Fondacaro et al., 1990). Procedures in this study were modified from previously described experiments in the studies of intestinal transit in rodents (Al-Qarawi et al., 2003; Moon et al., 1979; Qin et al., 2003). Female CD rats aged 6–8 weeks were fasted for 18 to 24 h before treatment with linaclotide (1.25, 2.5, 5, 10, or 20 μg/kg) or vehicle, but allowed free access to water throughout the study. Treatments were administered to the animals (300 μl/animal) (n = 5–6/group) by gavage immediately before an oral dose (500 μl/animal) of a 10% activated carbon /10% gum arabic (Sigma, St. Louis, MS) suspension in water. After 10 min, the rats were sacrificed and their intestines from the stomach to the cecum were removed. Total length of the intestine was measured for each animal in addition to the distance traveled from the stomach by the charcoal front. Studies of the same design were also conducted in male CD rats.

2.14. Statistics

Statistical significance between independent samples was determined using the Student's unpaired two-tailed *t* test. The significance in the rat gastrointestinal transit study was determined using analysis of variance (ANOVA), followed by an unpaired two-tailed Student's *t* test. All data are expressed as the mean ± standard error of the mean (S.E.M.). A P value <0.05 is considered statistically significant.

3. Results

3.1. Determination of the solution structure of linaclotide by nuclear magnetic resonance

The accurate mass of linaclotide, determined using LC-TOF/MS is 1525.4, consistent with all six cysteines in the molecule involved in disulfide bonds. This observation was confirmed using 2D ^{1}H-^{13}C-HSQC NMR spectrum of linaclotide. The ^{13}C chemical shifts of the C^{β} from the six cysteines of linaclotide were between 34 and 43 ppm, which is

consistent with the cysteines being in the oxidized form. The analysis of a database of measured oxidized cysteine ^{13}C C^{β} chemical shifts indicates that most have chemical shifts of 34 to 51 ppm (Sharma and Rajarathnam, 2000).

The 3D structure of linaclotide was determined by first obtaining the upper distant limits derived from the NOESY spectrum using automated NOESY assignments and iterative assignment/structure calculation cycles. Fig. 1A shows a superimposition of distance–geometry structures of linaclotide calculated using the NOE data. Fig. 1B shows the structure of linaclotide after restrained energy minimization. The linaclotide molecule is folded as a compact right-handed spiral with three turns that are fixed in place by three disulfide linkages. The structure calculated by NMR spectroscopy is consistent with disulfide bonds between Cys^1 and Cys^6, Cys^2 and Cys^{10}, and Cys^5 and Cys^{13} (Fig. 1C). This disulfide pattern is conserved between linaclotide and STa, and with the two disulfide bonds in guanylin and uroguanylin.

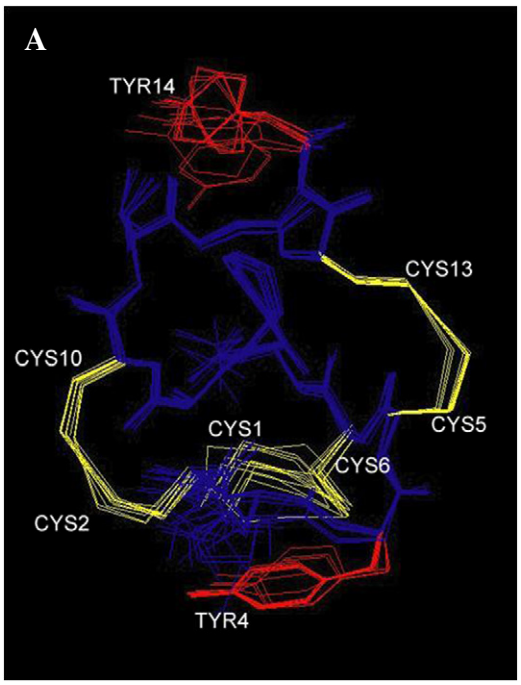
3.2. Linaclotide binding to intestinal guanylate cyclase C receptors

Using mice genetically deficient in the guanylate cyclase C gene, we have previously shown that this transmembrane receptor is the molecular target of linaclotide (Bryant et al., 2010). The affinity of linaclotide for guanylate cyclase C receptors on human colon carcinoma T84 cells, known to express this receptor, was measured using a competitive radioligand binding assay with [^{125}I]-STa. Linaclotide inhibited [^{125}I]-STa binding to T84 cells in a concentration-dependent manner at pH 7.0 (Fig. 2A). The radioligand binding data were analyzed using computer software and fitted to curves consistent with a two-site binding model (Hamra et al., 1997), resulting in a K_i for linaclotide of 1.24 ± 0.63 nM for the high affinity site and 122 ± 35 nM for the low affinity site. The binding affinity of STa was also determined under the same conditions, resulting in a calculated K_i of 1.57 ± 1.1 nM for the high affinity site and $\approx$446 nM for the low affinity site. Linaclotide also inhibited [^{125}I]-STa binding to guanylate cyclase C receptors on rat intestinal mucosal cells in a concentration-dependent manner, with a K_i of 4.2 ± 0.98 nM similar to that measured for T84 cells (Fig. 2B). The binding curve of [^{125}I]-STa to rat intestinal mucosal cells in the presence of increasing concentrations of linaclotide best fit a one-site model.

The binding affinities of linaclotide, guanylin and uroguanylin to human guanylate cyclase C at a variety of pH conditions found in the gastrointestinal tract are summarized in Table 1, and the corresponding binding curves are shown in Fig. 2C, D, E. The binding affinity of linaclotide to guanylate cyclase C receptors on T84 cells at pH 5 and pH 8 is similar to that at pH 7 (Table 1). In contrast, the binding affinity of guanylin is decreased at pH 5 compared to pH 8 while the binding of uroguanylin is decreased at pH 8 compared to pH 5, consistent with previous observations (Hamra et al., 1997). In this study, all binding curves were fit to a one-site model, using the GraphPad software program.

3.3. Effects of linaclotide, guanylin, and uroguanylin stimulation of guanylate cyclase C receptors on cGMP accumulation in human T84 cells

Ligand binding to guanylate cyclase C receptors stimulates the intrinsic guanylate cyclase activity of the receptor, resulting in the conversion of guanosine 5′-triphosphate to cGMP and increased intracellular levels of this signalling molecule (Huott et al., 1988). As shown in Fig. 3, linaclotide stimulates the accumulation of cGMP in human T84 cells in a concentration-dependent manner and caused a significant ($P<0.05$) increase of cGMP levels compared to those produced by either guanylin or uroguanylin, at concentrations of $\geq$ 1 nM (Fig. 3). The concentration of guanylate cyclase C agonist which produced 50% of the maximal activity (EC_{50}) of linaclotide at pH 7.0 (99 nM $\pm$ 17.5 nM) was 8 to 10-fold more potent than that of guanylin



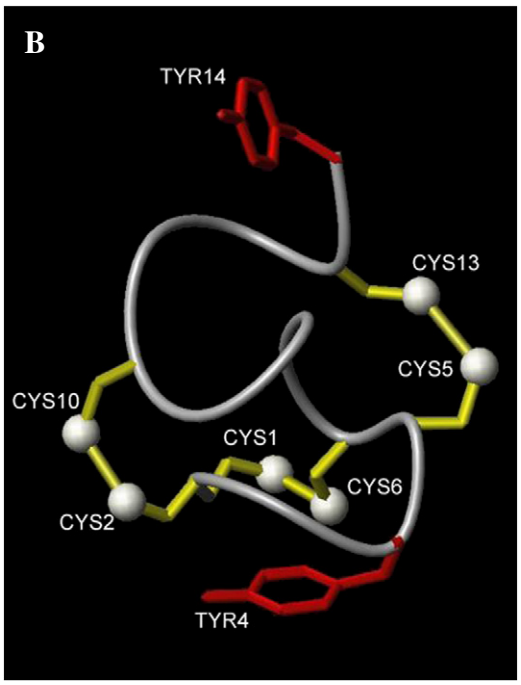


Fig. 1. Solution structure of linaclotide and amino acid sequence of linaclotide**.** A, best fit superposition of the backbone atoms of the final structure of linaclotide. Backbone atoms are colored purple, the C^{β} and S^{γ} atoms of cysteine residues are colored yellow, and the two side chains of tyrosine are colored red. B, diagram of energy minimized structure of linaclotide. The backbone is represented as a grey tube, the C^{β} of cysteine are shown as yellow sticks, and the S^{γ} atoms of cysteine are shown as small spheres. C, the cysteine residues in the amino acid sequence of linaclotide involved in the formation of intramolecular disulfide bonds in the pharmacologically active conformation of linaclotide are connected by lines.

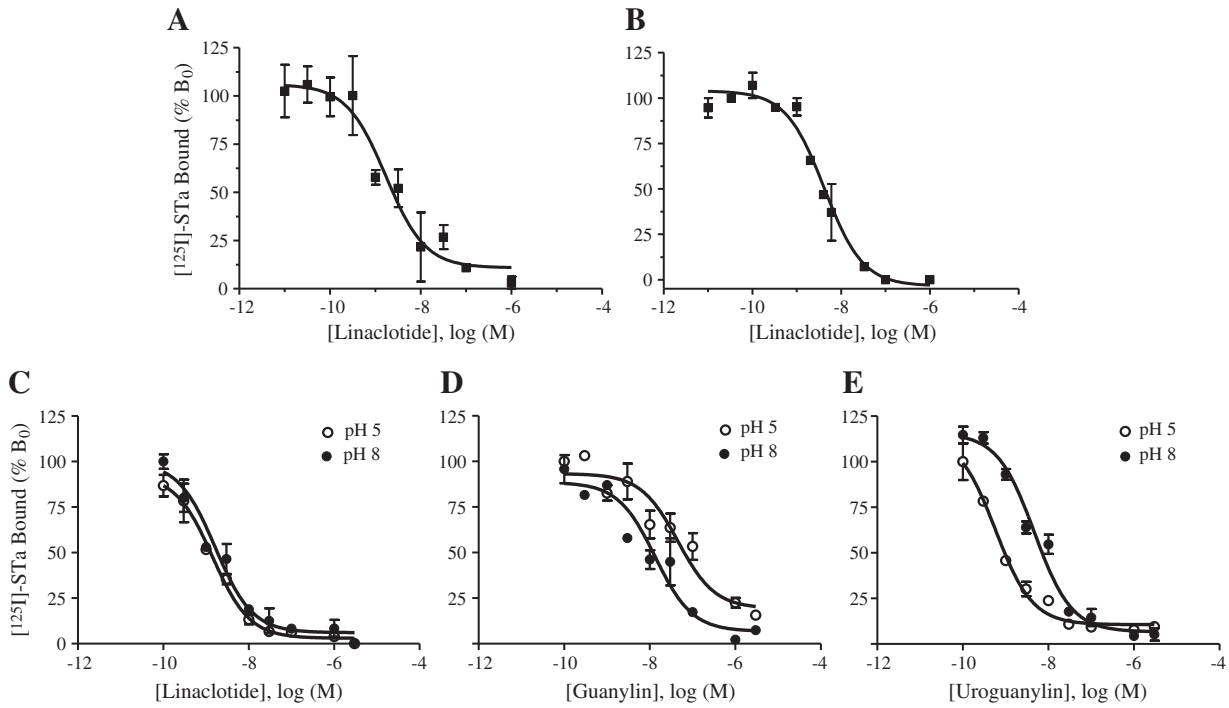


Fig. 2. Competitive radioligand binding assays of linaclotide, guanylin and uroguanylin at varying pH conditions. A, linaclotide binding to guanylate cyclase C receptors on T84 cells (pH 7.0). Cells were incubated for 1 h at 37 °C with [^{125}I]-STa and increasing concentrations of linaclotide. B, linaclotide binding to guanylate cyclase C receptors on rat intestinal mucosal cells (pH 7.0). Cells were incubated for 1 h at 25 °C with [^{125}I]-STa and increasing concentrations of linaclotide. C, D, E, binding of [^{125}I]-STa to guanylate cyclase C receptors on T84 cells was determined in the presence of the indicated concentrations of linaclotide (C), guanylin (D), or uroguanylin (E) at pH 5 and pH 8 after 1 h incubation at 37 °C. Specific binding (%) was obtained by dividing the specifically bound [^{125}I]-STa at each linaclotide, guanylin or uroguanylin concentration by the specifically bound [^{125}I]-STa in the absence of either peptide (B_0). Each point represents the mean of two independent binding experiments. All data are expressed as the mean ± S.E.M.

or uroguanylin at pH 7.0 (970 nM ± 236 nM and 798 nM ± 217 nM) in this assay and statistical analysis of comparisons of linaclotide versus guanylin and linaclotide versus uroguanylin using the F-test showed that the EC_{50} of linaclotide is significantly different from those of guanylin and uroguanylin ($P<0.0001$).

3.4. Stability of linaclotide in acidic pH and resistance to digestion by gastric hydrolases

The transport of pharmacologically relevant concentrations of orally administered linaclotide into the intestinal lumen could potentially be prevented by instability and inactivation of this peptide during its passage through the stomach. Therefore, we determined the stability of linaclotide in vitro under conditions mimicking the gastric environment. No degradation of linaclotide was observed after 3 h of incubation in simulated gastric fluid (pH 1), compared to the linaclotide control in distilled water. Similarly, linaclotide was completely resistant to enzymatic hydrolysis by pepsin after 3 h of incubation (data not shown). In contrast, insulin B chain oxidized, a known substrate of pepsin was efficiently hydrolyzed (~50% hydrolysis after 1 h) by pepsin under identical conditions (data not shown).

Table 1
Binding affinities of linaclotide, guanylin and uroguanylin on T84 cells under different pH conditions. K_i values are calculated from radioligand competition curves and are indicated as mean ± S.E.M.

Competitor	K_i, [nM]
Linaclotide, pH 7	1.23 ± 0.63
Linaclotide, pH 5	1.41 ± 0.29
Linaclotide, pH 8	1.64 ± 0.46
Guanylin, pH 5	45.4 ± 18.7
Guanylin, pH 8	13.4 ± 4.75
Uroguanylin, pH 5	0.58 ± 0.12
Uroguanylin, pH 8	4.72 ± 0.94

Abbreviations: K_i, dissociation equilibrium constant for inhibitor binding; IC_{50}, half-maximal inhibitory concentration.
Since the [^{125}I]-STa concentration (170 pM) used in this assay was very small when compared to its dissociation constant (15 nM), the calculated K_i is effectively identical to the IC_{50}.

3.5. Oral bioavailability of linaclotide in rats

We measured the oral bioavailability of linaclotide in rats to investigate whether potential pharmacological effects elicited in vivo in the intestinal lumen could be associated with a mechanism(s) that is dependent on systemic availability of this peptide. Following a 10 mg/kg intravenous or oral dose, linaclotide was quantified up to 6 h (Fig. 4). Pharmacokinetic analysis showed that linaclotide was only minimally

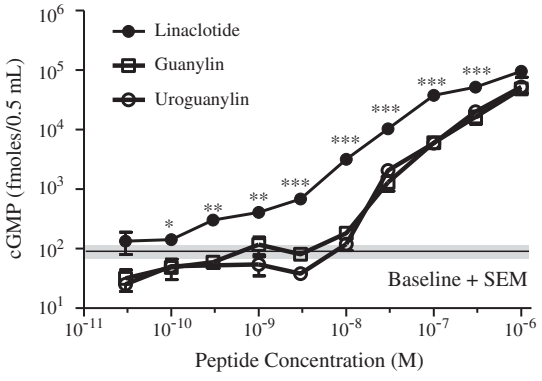


Fig. 3. Accumulation of cGMP in T84 cells exposed to linaclotide, guanylin and uroguanylin. Cells were incubated for 30 min with increasing concentrations of linaclotide, guanylin, or uroguanylin. The results represent the mean of four independent assays. Statistical significance at 0.1 and 0.3 nM determined versus guanylin, not determined versus uroguanylin. All data are expressed as the mean ± S.E.M. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus guanylin and uroguanylin.

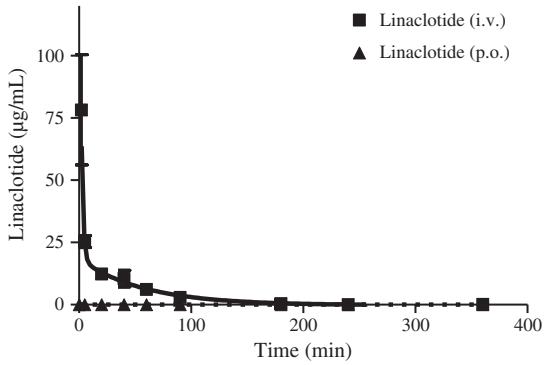


Fig. 4. Oral bioavailability of linaclotide in rats. Linaclotide was administered either intravenously (10 mg/kg) or by oral gavage (10 mg/kg), and the plasma concentration of linaclotide was determined at specific time intervals by LC/MS. The limit of detection of linaclotide in plasma was 0.1 nM. All data are expressed as the mean ± S.E.M.; n = 3/timepoint.

absorbed. The area under the curve $(AUC)_{i.v.(0–6\ h)}$ was 18,800 ng-h/ml, the $AUC_{p.o.(0–6\ h)}$ was 19.7 ng-h/ml, and the resulting oral bioavailability (%F) was 0.1%.

3.6. Effects of linaclotide on gastrointestinal transit in rats

The dose-dependent effects of acute oral doses of linaclotide on gastrointestinal transit were assessed in CD rats. The distance traveled by the charcoal front in female rats after 10 min, expressed as a percent of total length of small intestine, is shown in Fig. 5. All groups that received linaclotide at doses of 5, 10 and 20 μg/kg displayed a statistically significant increase in the gastrointestinal transit rate compared to vehicle-treated animals ($P<0.05$). Similar effects of linaclotide on gastrointestinal transit rates were observed in male CD rats (data not shown).

3.7. Effects of linaclotide on fluid secretion and cGMP accumulation in rat small intestinal loops

We further assessed the activation of intestinal guanylate cyclase C receptors in vivo after injection of linaclotide (5 μg) into surgically ligated rat duodenal, jejunal and ileal loops. Guanylate cyclase C activation was estimated by measuring the luminal fluid content and cGMP levels after exposure to linaclotide or vehicle for 90 min. The volume of luminal fluid in ligated duodenal, jejunal and ileal loops significantly increased in response to linaclotide treatment, compared to the volume of fluid from vehicle-treated animals (Fig. 6A). This stimulation of fluid secretion after treatment with linaclotide was

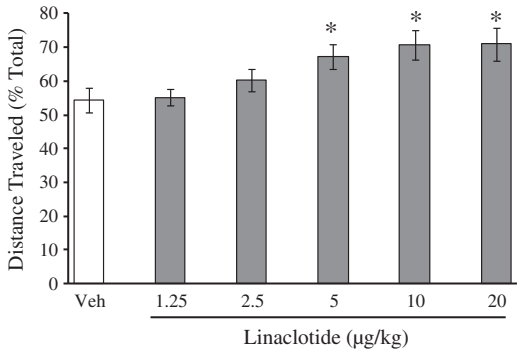


Fig. 5. Orally administered linaclotide accelerates gastrointestinal transit in rats. Female rats (n = 5-6/group) were dosed orally with the indicated doses of linaclotide, immediately prior to an oral dose of the indicator 10% activated carbon suspension. After 10 min, the rats were sacrificed and the distance traveled by the dye was measured. All data are expressed as the mean ± S.E.M. *$P<0.05$ versus vehicle.

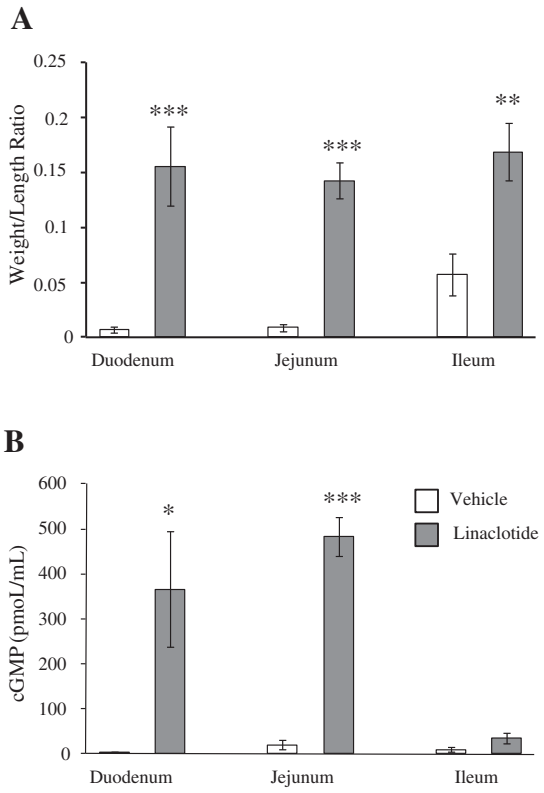


Fig. 6. Linaclotide stimulates the secretion of fluid and cGMP into rat intestinal loops. A, ligated loops were surgically introduced into the duodenum, jejunum and ileum, and linaclotide (5 μg) or vehicle was injected into these loops. After 90 min, the rats were sacrificed, loops excised and the weight to length (W/L) ratio was determined. B, the concentration of cGMP in these loops after 90 min exposure to linaclotide or vehicle was measured using a competitive enzyme immunoassay. All data are expressed as the mean ± S.E.M.; n = 10. **$P<0.01$, ***$P<0.001$ versus vehicle.

accompanied by a significant increase in concentrations of secreted cGMP in the duodenum and jejunum, but a less profound increase in ileal loops, from the same linaclotide-treated animals (Fig. 6B). However, the linaclotide-stimulated increase in cGMP secretion in the ileum was still approximately 3.5-fold higher when compared to vehicle-treated animals, although it did not reach statistical significance ($P = 0.051$). This nonuniform distribution of cGMP secretion in vitro and in vivo into rat small intestinal loops has previously also been reported in response to exposure to STa, specifically the relative insensitivity of the ileum (London et al., 1997; Qian et al., 2000). These results indicate that linaclotide treatment increases cGMP levels and induces fluid secretion into the lumen of the rat small intestine, pharmacological effects consistent with the stimulation of guanylate cyclase C receptors in the rat small intestine.

4. Discussion

Linaclotide is a first-in-class, orally administered 14-amino acid peptide that is in development for the treatment of IBS-C and chronic constipation. Although linaclotide has disulfide linkages and sequence motifs similar to guanylin and uroguanylin and thus should therefore be classified as a member of the guanylin family of guanylate cyclase C agonist peptides, it possesses a number of important pharmacological properties that clearly distinguishes it from the endogenous peptide hormones.

First, we determined the pharmacologically active conformation of linaclotide and found that this peptide is folded as a compact right-handed spiral with three turns that are fixed in place by disulfide linkages. This structure is consistent with the involvement of all six cysteine residues in the formation of three intramolecular disulfide

bonds between cysteines 1 and 6, 2 and 10, and 5 and 13, similar in structure to the guanylate cyclase C agonists guanylin and uroguanylin (isoforms A), and microbial STa peptides (Sato et al., 1994; Skelton et al., 1994). However, in contrast to the three intramolecular disulfide bonds present in linaclotide and microbial STa peptides, only two such bonds are formed in the physiologically active structures of guanylin and uroguanylin. Consistent with these findings, the reported rank order for guanylate cyclase C agonist potency: STa>guanylin/uroguanylin>lymphoguanylin (lymphoguanylin contains a single disulfide bond) suggests that increased agonist potency is linked to the stabilization of the physiologically active structures of these peptides through multiple disulfide bonds. Therefore, the compact molecular structure of pharmacologically active linaclotide is anticipated to result in increased resistance to proteolysis in the gastrointestinal lumen, resulting in prolonged exposure to guanylate cyclase C receptors.

Second, linaclotide displayed comparable binding affinities to guanylate cyclase C receptors across the broad range of physiological pH conditions (pH 5–8) found in the gastrointestinal tract in studies using human T84 colon carcinoma cells. These binding affinities are similar to those exhibited by guanylin and uroguanylin, at each of their optimal pH conditions, which are known to regulate their differential activity in different regions of the intestine. This pharmacological property distinguishes linaclotide from guanylin and uroguanylin and suggests that the pH-independence of linaclotide binding to guanylate cyclase C receptors supports the ability of this peptide to help modulate intestinal fluid homeostasis along the whole longitudinal axis of the gastrointestinal tract independent of locally varying pH environments.

While the binding curve of linaclotide to guanylate cyclase C receptors on T84 cells was consistent with a two-site model previously described for guanylate cyclase C agonist peptides, binding to rat intestinal mucosa cells best fit a one-site model. The two binding sites on T84 cells could constitute either interchangeable high and low affinity states of the guanylate cyclase C receptor or a second, low affinity non-guanylate cyclase C receptor binding site (Rao et al., 2004; Sellers et al., 2008). Our findings support a model wherein guanylate cyclase C is the principal receptor for linaclotide and more broadly, guanylin family peptide-mediated regulation of intestinal fluid homeostasis.

Third, linaclotide binding to guanylate cyclase C receptors on T84 cells in vitro was accompanied by a concentration-dependent, significant increase in the intracellular levels of the second messenger cGMP, compared to guanylin and uroguanylin, consistent with the rank order: linaclotide>guanylin/uroguanylin reported for guanylate cyclase C-binding of these peptides. The elevation of cGMP is known to be involved in the activation of the cystic fibrosis transmembrane conductance regulator (Schlossman et al., 2005; Vaandrager et al., 1998) which results in an increase in chloride and bicarbonate secretion in the intestinal lumen (Field et al., 1989; Forte, 1999; Rao et al., 2004), accompanied by a decrease in water and sodium absorption across the intestinal epithelium (Cha et al., 2005; Sindic and Schlatter, 2006). The physiological effect of guanylate cyclase C stimulation therefore results in an increase in intestinal fluid. Thus, based on our findings it is anticipated that orally administered linaclotide could activate guanylate cyclase C receptors throughout the gastrointestinal tract, thereby increasing intestinal transit and fluid secretion from the pyloric portion of the stomach through the colon.

The presence of pharmacologically relevant concentrations of active linaclotide in the intestine after oral administration is dependent on its stability in conditions encountered during its passage through the stomach, specifically the exposure to a highly acidic pH and gastric hydrolases. Following several hours of incubation, linaclotide was stable in simulated gastric fluid (pH 1) and proved completely resistant to hydrolysis by pepsin, suggesting that pharmacological active linaclotide persists in the intestinal tract.

Although guanylate cyclase C receptors are predominantly expressed on the luminal surface of intestinal epithelial cells, the target cell population for linaclotide in the intestinal tract, we performed pharmacokinetic studies to measure the oral bioavailability of linaclotide to determine whether the activity of linaclotide in vivo could potentially be related to mechanism(s) which are dependent on the systemic availability of this peptide. Our estimated rat oral bioavailability of 0.1% supports the hypothesis that the pharmacological activity of linaclotide is locally driven in the gastrointestinal tract.

The primary clinical symptoms of irritable bowel syndrome are chronically recurring abdominal pain and discomfort, associated with altered bowel habits. Since altered intestinal transit time shows evidence of correlation with irritable bowel syndrome symptoms in the clinic (Bouchoucha et al., 2006), we assessed the effects of linaclotide in vivo in rat models of gastrointestinal transit and intestinal secretion. We found that orally administered linaclotide caused a dose-dependent, significant increase in the distance traveled by the charcoal front. Moreover, fluid volumes in ligated loops were significantly increased after exposure to linaclotide, and this increase in fluid secretion was accompanied by a significant increase in secreted cGMP into the luminal fluid of the duodenum and jejunum, but not the ileum. The limited secretion of cGMP in the ileum is potentially related to posttranslational regulation of guanylate cyclase C, which uncouples agonist binding from cyclase activity in this part of the intestine (Qian et al., 2000). This differential regional response pattern of cGMP secretion is evolutionarily conserved and has been found across multiple species, including opossum, turkey, duck and alligator, suggesting that the limited activation of guanylate cyclase C in the ileum is sufficient to maintain fluid homeostasis in this region of the intestine (Krause et al., 1994, 1995, 1997). Thus, it is conceivable that even the moderate elevation of cGMP levels in the ileum following exposure to linaclotide is sufficient to account for the observed significantly elevated fluid secretion into the lumen. Moreover, studies by Qian et al. have shown neither differential phosphodiesterase activity such as delayed cGMP hydrolysis nor differential rates of cGMP export in the ileum, lending further support to the hypothesis that the moderate elevation of cGMP levels in the ileum is the underlying mechanism for the observed fluid secretion (Qian et al., 2000).

These pharmacological responses of linaclotide in vivo further support the hypothesis of local activation of intestinal guanylate cyclase C receptors as the underlying mechanism of the pharmacological effects of this peptide, and also appear to exclude the involvement of smooth muscle cells, since guanylate cyclase C receptors are not expressed on smooth muscle cells and linaclotide has very low systemic exposure. Clinical studies have shown that slower gastrointestinal transit times are more likely to be seen in irritable bowel syndrome with constipation patients and gastrointestinal transit measures in these patients probably have the best predictive value for specific irritable bowel syndrome symptoms, such as stool form and ease of defecation (Bouchoucha et al., 2006). Therefore, the pharmacological effects of linaclotide in rat models of gastrointestinal function suggest that this agent may have the therapeutic potential to reduce some of the most bothersome symptoms in irritable bowel syndrome with constipation and chronic constipation patients, including infrequent and hard stools, straining, and incomplete evacuation. This was confirmed in clinical studies with orally administered linaclotide that have demonstrated improved bowel habits, abdominal symptoms, and global relief in chronic constipation patients (Lembo et al., 2010). Moreover, a recent study that investigated the antinociceptive properties of orally administered linaclotide in several mechanistically different rodent models of inflammatory and non-inflammatory visceral pain found that this peptide significantly decreased visceral pain in these models, in a guanylate cyclase C receptor-dependent manner (Eutamene et al., 2009). These results suggest that the pharmacological effects elicited by linaclotide may also be capable of reducing abdominal pain in patients suffering from

irritable bowel syndrome with constipation and other functional gastrointestinal disorders.

In conclusion, we provide evidence that linaclotide is a potent guanylate cyclase C agonist that, through pH-independent, high affinity binding to guanylate cyclase C receptors, stimulates the production and secretion of cGMP from intestinal epithelial cells. This peptide is stable under conditions that mimic the gastric environment and has very low oral bioavailablity. Moreover, linaclotide elicits significant pharmacological responses locally in the gastrointestinal tract in rat models of gastrointestinal function, including increased intestinal fluid secretion and accelerated gastrointestinal transit. Taken together, these physical and pharmacological properties suggest that orally administered linaclotide may be capable of improving bowel habits in patients suffering from irritable bowel syndrome with constipation, chronic constipation and other functional gastrointestinal disorders.

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