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Pharmacological Properties, Metabolism and Disposition of Linaclotide, a Novel Therapeutic Peptide Approved for the Treatment of Irritable Bowel Syndrome with Constipation and Chronic Idiopathic Constipation[#]

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Running Title: Pharmacology, Metabolism, and Disposition of Linaclotide

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Abstract

Linaclotide, a potent guanylate cyclase C agonist, is a therapeutic peptide approved in the United States for the treatment of irritable bowel syndrome with constipation and chronic idiopathic constipation. Here we present for the first time the metabolism, degradation, and disposition of linaclotide in animals and humans. We examined the metabolic stability of linaclotide in conditions that mimic the gastrointestinal tract and characterized the metabolite MM-419447, which contributes to the pharmacological effects of linaclotide. Systemic exposure to these active peptides is low in rats and humans, and the low systemic and portal vein concentrations of linaclotide and MM-419447 observed in the rat confirmed both peptides are minimally absorbed following oral administration. Linaclotide is stable in the acidic environment of the stomach and is converted to MM-419447 in the small intestine. The disulfide bonds of both peptides are reduced in the small intestine, where they are subsequently proteolyzed and degraded. Following oral administration of linaclotide, < 1% of the dose was excreted as active peptide in rat feces and a mean of 3-5% in human feces; in both cases MM-419447 was the predominant peptide recovered. MM-419447 exhibits high-affinity binding in vitro to T84 cells, resulting in a significant, concentration-dependent accumulation of intracellular cGMP. In rat models of gastrointestinal function, orally dosed MM-419447 significantly increased fluid secretion into small intestinal loops, increased intra-luminal cGMP, and caused a dose-dependent acceleration in gastrointestinal transit. These results demonstrate the importance of the active metabolite in contributing to linaclotide's pharmacology.

Introduction

Peptide therapeutics represent an emerging class of drugs with significant pharmacological potential, due to their high specificity, affinity, and molecular target recognition (Yu et al., 2004). Linaclotide is a novel, orally administered 14-amino acid peptide approved for the treatment of irritable bowel syndrome with constipation (IBS-C) and chronic idiopathic constipation (CIC) (Wensel and Luthin, 2011; Lembo et al., 2011; Chey et al., 2012; Rao et al., 2012). Linaclotide is a potent guanylate cyclase C (GC-C) agonist and member of the guanylin family of peptides known to regulate cyclic guanosine-3',5'-monophosphate, or cGMP (Currie et al., 1992; Forte, 2004; Hamra et al., 1993) through their activity at GC-C. The endogenous peptide hormones guanylin and uroguanylin act on GC-C expressed on the luminal surface of intestinal epithelial cells (Wiegand et al., 1992; Fan et al., 1996; Hamra et al., 1996; Hamra et al., 1997; Schulz et al., 1990) to regulate intestinal fluid secretion in response to a meal. Linaclotide binds to GC-C with high affinity in a pH-independent manner, stimulates the intracellular production of cGMP, and elicits its potent pharmacological response locally in the gastrointestinal (GI) tract with very low oral bioavailability (Busby et al., 2010). Experiments in genetically modified mice lacking the gene encoding GC-C (*Gucy2*$^{-/-}$) have shown that this receptor is the molecular target of linaclotide (Bryant et al., 2010). In addition to increasing fluid secretion and accelerating GI transit, linaclotide has also been shown to have potent GC-C-mediated analgesic effects in several mechanistically different rodent models of visceral hypersensitivity (Eutamene et al., 2010). These distinct pharmacological effects on relieving pain and accelerating GI transit have been observed in the clinic (Andresen et al., 2007; Johnston et al., 2010). Phase III studies

have shown that linaclotide treatment results in a significant reduction in abdominal pain, discomfort, bloating and constipation symptoms in IBS-C patients (Roque and Camilleri, 2011; Chey et al., 2012; Rao et al., 2012) and improves bowel and abdominal symptoms in chronic constipation patients (Lembo et al., 2010). Thus, GC-C appears to be a promising therapeutic target for the treatment of functional gastrointestinal disorders, such as IBS-C and CIC.

Orally administered peptides typically have low absolute oral bioavailability, therefore achieving systemic plasma concentrations is often a challenge; however, this is an advantage with linaclotide, as the ligand-binding domain of the GC-C target is located on the luminal surface of intestinal epithelial cells and systemic exposure is not necessary for linaclotide to exert its pharmacological effects. Effective pharmacotherapy with linaclotide depends on the local concentrations present within the intestinal tract. This therapeutic goal is challenged by the varied and harsh environments along the gastrointestinal tract, which decrease the amount of active peptide available. These environments include vastly different pH conditions ranging from pH 2 in the stomach up to pH 8 in the duodenum, as well as proteolytic enzymes such as gastric hydrolases (pepsins), pancreatic hydrolases (trypsin, chymotrypsin, elastase, aminopeptidases, and carboxypeptidases A and B), and intestinal brush border membrane-bound enzymes (carboxypeptidases, endopeptidases and aminopeptidases) which are also involved in peptide degradation (Johnson et al., 1994; Wilkinson et al., 1997; Sanderick et al., 1988; Woodley et al., 1994). This extensive system of presystemic metabolism and degradation has evolved in order to digest ingested proteins and large peptides into small, absorbable di- and tripeptides and single amino acids (Daniel, 2004). In addition to pH instability

and/or proteolytic degradation, peptide drugs containing disulfide bonds are inactivated by the reduction of these bonds in the GI tract. These reactions are carried out by the glutaredoxin/glutathione reductase and thioredoxin/thioredoxin reductase systems, an integral part of the antioxidant defense system in the GI tract (Fernandes and Holmgren, 2004; Avval and Holmgren, 2009).

In the studies described here, we have investigated the pharmacological properties, metabolism, and disposition of linaclotide as well as the role the active metabolite plays in contributing to the pharmacological effects of orally dosed linaclotide.

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Materials and Methods

Linacotide, MM-421202 (CCEY), MM-421522 (CCNPACTGCY), and [$^{13}C_1$]-Ala-linaclotide were obtained from Polypetide Laboratories (Torrance, CA). MM-419447 (CCEYCCNPACTGC) was synthesized at Polypetide Laboratories. (Wolfenbuettel, Germany); MM-420026 ([$^{15}N^{13}C_9$]-linaclotide), MM-437150 ([$^{15}N^{13}C_9$]-MM-419447), and human heat-stable enterotoxin (hSTa) were synthesized at American Peptide Company (Sunnyvale, CA). Human guanylin was purchased from Peptide Institute, Inc. (Osaka, Japan). Bovine insulin, pepsin, chymotrypsin, carboxypeptidase A, pancreatin, and iodoacetamide were obtained from Sigma (St. Louis, MO). Trypsin was purchased from Promega (Madison, WI) and aminopeptidase was purchased from EMD Chemicals (Gibbstown, NJ). Heat-stable enterotoxin from porcine origin (pSTa) was purchased from Sigma and Bachem Americas (Torrance, CA) and radioiodinated at Perkin-Elmer Life and Analytical Sciences (N. Billerica, MA). Iodination of pSTa produces two monoiodinated molecules; the one labeled at Tyr^4 (Thompson et al., 1985) was purified by HPLC and used for radioligand binding competition studies, while the other, labeled at Tyr^{18}, was used for radioimmunoassay (specific activity 2,200 Ci/mmol). Monoclonal antibodies to pSTa were a gift of Dr. Ralph Giannella, University of Cincinnati (Brandwein et al., 1985). Bovine serum albumin (BSA) was obtained from Fisher Scientific. Sheep anti-mouse IgG beads were obtained from Invitrogen (Carlsbad, CA).

Animals. Male and female CD rats were obtained from Charles River Laboratories (Wilmington, MA) and housed in an environmentally controlled room. All animal studies were approved by the Ironwood Pharmaceuticals Institutional Animal Care and Use Committee.

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Stability of Linaclotide In Vitro. The methods used to study the stability of linaclotide in simulated gastric fluid and in the presence of pepsin, trypsin, chymotrypsin, aminopeptidase, and carboxypeptidase A are described in Supplemental Methods.

Determining Systemic and Portal Vein Concentrations of Linaclotide and MM-419447 Following Oral Administration of Linaclotide to Female Rats. Two cohorts consisting of four female Sprague-Dawley rats each were used in the study; one group (Group 1) was equipped with surgically implanted jugular vein cannulas, while the second group (Group 2) was equipped with portal vein cannulas. All animals received a single oral gavage of 10 mg/kg of linaclotide and systemic blood samples were collected from Group 1 via the jugular vein cannulas prior to dosing and 0.08 (5 min), 0.33 (20 min), 0.67 (40 min), 1, 1.5, 3, 4.5, 6, and 24 hours after dosing. Portal vein blood samples were collected from the animals in Group 2 at the same designated time points via the portal vein cannulas. Plasma was extracted from the blood samples and analyzed for linaclotide and MM-419447 using liquid chromatography with tandem mass spectrometry (LC/MS/MS) as described in Supplementary Methods.

Determination of the Absolute Oral Bioavailability of MM-419447 in Female Rats. To determine absolute oral bioavailability of MM-419447, four groups (n = 3) of female rats received MM-419447 (10 mg/kg) intravenously (i.v.), while four groups (n = 3) received MM-419447 (10 mg/kg) by oral gavage (p.o.). Blood was drawn by retro-orbital eye bleeding at specific time intervals and frozen for subsequent LC/MS/MS analysis as described in Supplemental Methods. The concentration of MM-419447 was determined from peak response relative to a set of standards, and these concentrations were plotted as a function of time using GraphPad Prism 4.0 software. The limit of

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detection for MM-419447 in rat plasma was 1 ng/ml. Oral bioavailability (F) was calculated using the equation:

$F = (AUC_{p.o.(0\text{-}6h)} \times D_{i.v.}) / (AUC_{i.v.(0\text{-}6h)} \times D_{p.o.})$, where $D_{i.v.}$ and $D_{p.o.}$ equal the intravenous and oral dose, respectively.

Method for Quantitative Determination of Linaclotide and MM-419447 in Human Plasma. Linaclotide and MM-419447 were extracted from human plasma 2 h after oral dosing of linaclotide (see: Supplemental Methods). The plasma was analyzed for linaclotide and MM-419447 concentrations using LC/MS/MS as described above.

Recovery of Linaclotide and MM-419447 from Rat Feces After Oral Dosing. Three female CD rats (Charles River, Wilmington, MA) weighing approximately 180 g were included in this study. Three hundred µl of a 6 mg/ml solution of linaclotide (in 20 mM Tris-HCl, pH 7.5) was administered orally to all rats using a disposable feeding needle. Animals were individually housed and placed on wire grids in lined cages to ensure that all feces were collected. Feces were collected every hour over 55 hours after dosing. The total weight of feces collected at each time point was recorded. The feces were stored at -20°C until analysis. The concentrations of linaclotide and MM-419447 in feces were determined by LC/MS/MS as described in Supplementary Methods.

Extraction of Linaclotide and MM-419447 from Human Feces. Linaclotide and MM-419447 were extracted from human feces using a Bond Elute Plexa solid phase extraction (SPE) plate. Approximately 15 g of sample was homogenized in a blender with three volumes of deionized water (w/v) containing internal standard (MM-420026). The supernatant of a 2 g aliquot from each sample was filtered through a Whatman 0.2 µm PVDF w/GMF syringe filter and loaded onto the SPE plate. The samples were washed

with 5% methanol in water and eluted from the plate with 100% methanol. The samples were evaporated under nitrogen and reconstituted in 20% acetonitrile and 0.1% formic acid in water. A 40-µl injection of the extract was analyzed with an LC/MS/MS assay using a Hypersil Gold column (3-µm particle, 2.1 × 50 mm). The HPLC method utilized a binary buffer system with 5% acetonitrile and 0.1% formic acid in water as the weak solvent (A) and 20% acetonitrile and 0.1 % formic acid in water as the strong solvent (B). The HPLC gradient started with 50% B for 4 min and ramped down to 10% over the next 3.5 min and returned back to 50% B after 30 seconds. The analytes were detected by an MRM (multiple reaction monitoring) method using an Applied Biosystems API 5000 mass spectrometer operating in positive ion electrospray ionization mode. The method monitored the transitions 764>182 for linaclotide, 682>136 for MM-419447, and 769>192 for MM-420026 (IS). The peak area ratios of linaclotide/IS and MM-419447/IS and the theoretical concentrations of calibration samples were fitted to the quadratic function with $1/x^2$ weighting, excluding the origin.

Metabolism of Linaclotide In Vivo and In Vitro. The stability and metabolism of linaclotide was studied in vivo in surgically ligated intestinal loops and in vitro in intestinal fluid extracted from the small intestine of rodents and humans as described in Supplemental Methods.

Competitive Radioligand Binding Assays Using Human T84 Cells

Competitive radioligand binding assays using T84 cells and [^{125}I]-pSTa were conducted as described by Busby et al. (2010). Competitive radioligand binding assays using rat intestinal brush border membranes were conducted as described in Supplemental Methods.

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Cyclic GMP Accumulation in T84 Cells and in Fluid of Ligated Intestinal Loops. Accumulation of cGMP in T84 cells was conducted as described by Busby et al. (2010).

To assess the biological activity of linaclotide and MM-419447 when fully reduced, both peptides were incubated in the presence of 5 mM DTT for 30 min at 37°C followed by alkylation with 50 mM iodoacetamide for 30 min at room temperature in the dark. The fully reduced and alkylated samples were then purified using SPE to remove excess DTT and iodoacetamide, quantitated using UPLC-HRMS (described in Supplemental Methods) and subjected to T84 cells grown in 96 well plates (250,000 cells/well) and compared to fully oxidized parent peptides. Cyclic GMP was quantified using LC/MS/MS.

To measure the amount of cGMP in fluid obtained from ligated rat intestinal loops (*n*=10), the samples were immediately added to 6% ice-cold TCA at a ratio (v/v) of 300 µl of TCA to 100 µl of fluid sample and stored at -20°C until assayed. After thawed on ice, the samples were homogenized for 30 seconds and the precipitate was removed by centrifugation (1500 x *g*, 10 min). TCA was extracted from each sample with water-saturated ether and the amount of cGMP in intestinal fluid samples was determined with a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). All results are representative of several independent experiments.

Intestinal Fluid Secretion. Female CD rats (*n*=10/group) were used to generate intestinal loops (1-3 cm in length) in the duodenum, jejunum, and ileum (prepared as described above). Loops were injected with either 200 µl of MM-419447 (5 µg), linaclotide (5 µg), or vehicle (KRGH), and the animals were allowed to recover for 90 min prior to sacrifice. The loops were excised and the length and weight of each intestinal

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loop was recorded both prior to and after collection of the intestinal fluid content. A weight-to-length ratio (W/L) was calculated and expressed as a percentage of the control value, a commonly used surrogate to measure intestinal secretion (Thiagarajah et al., 2004).

Gastrointestinal Transit in Rats. A well-established model was used to measure gastrointestinal transit in the upper gastrointestinal tract, since measurements of colonic transit in rats have proven difficult due to the reservoir function of the cecum during periods of intestinal hypersecretion (Fondacaro et al., 1990). Female CD rats 6 to 8 weeks of age were fasted for 18 to 24 hr prior to treatment, but had access to water *ad libitum* during this period. The rats (n=10/group, except the 6.25 µg/kg group of n=5) were administered oral doses (300 µl) of either MM-419447 (6.25, 12.5, 25, 50 µg/kg), linaclotide (6.25, 12.5, 25, 50 µg/kg), or vehicle (20 mM Tris-HCl, pH 7.5) immediately prior to an oral dose of 10% activated carbon/10% gum arabic powder suspension (500 µl in water). After 10 min, the animals were euthanized and their intestines, from the pyloric junction to the cecum, were removed. The gastrointestinal transit is expressed as the percentage of the distance traveled by the charcoal front compared to the total length of the intestine. Studies following the same protocol were also performed in male CD rats.

Statistical Analysis. The determination of statistical significance of data was performed using either a one-way or two-way analysis of variance (ANOVA), followed by unpaired two-tailed Student's *t*-test or Wilcoxon and Mann-Whitney tests. All values are expressed as the mean ± standard error of the mean (SEM). A P value < 0.05 is considered statistically significant.

Results

Proteolytic and Acid Stability of Linaclotide in Vitro. Linaclotide was stable in simulated gastric fluid for at least 3 hours at 37°C, indicating that it is not degraded under the acidic conditions present in the stomach. Linaclotide was not digested during 3 hours of in vitro incubation with trypsin or pepsin, while insulin, a known substrate of these proteases, was digested. Similarly, linaclotide was not digested by aminopeptidase or chymotrypsin, while the control peptides (hSTa for aminopeptidase and guanylin for chymotrypsin) were rapidly hydrolyzed.

Incubation of linaclotide with carboxypeptidase A resulted in the formation of a single reaction product designated as MM-419447 (Figure 1). The rate of linaclotide loss under these conditions was equal to the rate of appearance of MM-419447 (Figure 1), with almost complete conversion of linaclotide to MM-419447 after 6 hours of digestion with carboxypeptidase A. To confirm the structure of MM-419447, the peptide was synthesized and shown to have the same retention time in UPLC as that of the peptide produced from linaclotide after digestion with carboxypeptidase A. The observed *m/*z ($[M + H]^+$) value for the MM-419447 product of linaclotide digestion with carboxypeptidase A was 1363.3333, and the observed *m/*z ($[M + H]^+$) value for MM-419447 synthesized by solid phase (1363.3319) are both within 1.8 ppm of the theoretical value predicted for this peptide (1363.3344). These observations identified MM-419447 as a peptide with an exact mass of 1362.3266 Da (elemental composition of $C_{50}H_{70}N_{14}O_{19}S_6$), corresponding to the metabolite of linaclotide that lacks the C-terminal Tyr^{14} residue. Carboxypeptidases digest proteins by cleaving the peptide bonds at C-terminal amino acids, resulting in the removal of C-terminal amino acid residues. In the

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case of linaclotide, under non-reducing conditions the cleavage of amino acids by carboxypeptidase A stops after the removal of the first C-terminal amino acid, Tyr^{14}.

Sensitivity of Linaclotide to Chymotrypsin and Pancreatin in the Absence and Presence of a Reducing Agent. Linaclotide in its native configuration was resistant to digestion by chymotrypsin, even though the peptide contains a tyrosine residue at the fourth position, which is a site predicted to be recognized by this protease. In addition, incubation of linaclotide with pancreatin, a mixture of proteases isolated from pig pancreas which is known to contain carboxypeptidase A (Bauer and Miskinis, 1947), resulted only in the generation of MM-419447. However, in the presence of a reducing agent (5 mM DTT), linaclotide was digested by chymotrypsin to two peptides (with sequences CCEY and CCNPACTGCY after cleavage of the C-terminal side of the Tyr^{4}) and to small peptide fragments by pancreatin.

Absolute Oral Bioavailability of MM-419447 in Rats. Previous pharmacokinetic studies showed that the oral bioavailability of linaclotide was 0.1% following the administration of a single 10 mg/kg dose (Busby et al., 2010). When MM-419447 was measured in rat plasma in the same experiment, very small amounts of this metabolite were found, demonstrating the generation of this metabolite in vivo. The combined oral bioavailability of linaclotide and MM-419447 was very low (0.13%), and the oral bioavailability of MM-419447 following administration of a single 10 mg/kg dose of MM-419447 was $\leq$ 0.1%, confirming that MM-419447 is a minimally absorbed peptide, similar to linaclotide (Table 1).

Systemic and Portal Vein Concentrations of Linaclotide and MM-419447 following Oral Administration of Linaclotide to Female Rats. After oral dosing to

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Sprague-Dawley rats, systemic exposure to linaclotide and MM-419447 was minimal, and the majority of the plasma, whether sampled from the jugular or portal vein, had concentrations of the test articles that were below the quantitation limits of 1 ng/ml (linaclotide) and 2 ng/ml (MM-419447) (Figure 2).

In systemic (jugular vein) circulation, the maximum observed linaclotide plasma concentration (C_{max}) in any animal was 2.97 ng/ml, and the time of the maximum observed plasma concentration (T_{max}) was 0.08 h (5 min). The corresponding highest individual portal vein C_{max} was 5.95 ng/ml and the T_{max} was 0.08 h (5 min). For the metabolite, the highest individual systemic C_{max} was 2.28 ng/ml and the highest individual portal vein C_{max} was 5.09 ng/ml. The metabolite T_{max} in both cases was 0.08 h (5 min). By 0.67 h (40 min), both systemic and portal vein plasma concentrations were below the lower limits of quantitation (LLOQ) for linaclotide and the metabolite, with the exception of animal 4, which had a systemic MM-419447 concentration of 2.80 ng/ml at 3 h. The systemic and portal vein pharmacokinetic profiles of linaclotide and MM-419447 following oral dosing are shown in Figure 2. Despite the high oral dose, concentrations in the jugular and portal veins were consistently low (≤ 6 ng/ml), indicating that the low absolute oral bioavailability of both peptides is due to poor absorption.

Recovery of Linaclotide and MM-419447 from Rat Feces after Oral Administration of Linaclotide. The total linaclotide recovered from the feces of rats 55 hours after oral dosing (10 mg/kg), as measured by LC/MS/MS, was 0.03% of the dose in the form of linaclotide and an additional 0.95% in the form of the metabolite MM-

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419447. In all rats tested, the majority of linaclotide and MM-419447 was recovered in the first 24 hours after oral dosing.

Plasma Concentrations of Linaclotide and MM-419447 in Humans after Oral Administration of Linaclotide. In a Phase 1, food-effect US study, plasma samples from fed and fasted subjects dosed with 290 µg of linaclotide once-daily for 7 days showed no quantifiable concentrations of linaclotide or MM-419447 (LLOQ = 0.2 ng/ml for linaclotide and LLOQ = 2.0 ng/ml for MM-419447). When linaclotide was administered as a single 10x dose (2,897 µg) on the eighth day, linaclotide concentrations were quantifiable in plasma from two of the 18 subjects (both fasted), and MM-419447 was not detectable in the plasma of any subject (Table 2). In the four pivotal Phase 3 trials in patients with IBS-C and CC, plasma was sampled on Day 1 and Day 29 in 465 out of 1,654 patients who received either 290 µg (*n*=313) or 145 µg (*n*=152) of linaclotide. Only two patients (both of whom received the 290 µg dose) had measurable plasma concentrations of linaclotide, each < 0.5 ng/ml, and none of the 465 patients had measurable plasma concentrations of MM-419447.

Recovery of Linaclotide and MM-419447 from Human Feces after Oral Administration. In the Phase 1 open-label two period cross-over food-effect study in healthy volunteers described in the previous section, stool samples were collected during the second period of dosing from fed and fasted subjects following oral administration of 290 µg of linaclotide once-daily for seven days, and then again following the administration of a single 10x dose (2,897 µg) on the eighth day. Recovery of active peptide ranged from 0 to 20% of the dose with a mean of 3-5% of dosed linaclotide. Virtually all of the recovered active peptide was MM-419447, confirming that it is a

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human metabolite (Table 3). Linaclotide was found in the stool samples from subjects only after administration of the 2,897 µg dose in the fed state; these three subjects had a median of 0.4% of dosed linaclotide in their stool. The amount of total recovered active peptide was similar in fed and fasted subjects (Table 3).

Metabolism of Linaclotide in Vivo in Surgically Ligated Rat Intestinal Loops. The metabolism of linaclotide in surgically ligated rat intestinal loops was studied as a function of time by injecting linaclotide in three locations (duodenum, mid-jejunum, and ileum). The fluid inside the loops was recovered at different times and linaclotide was extracted and quantified by radioimmunoassay. Linaclotide was not detectable within 5 min when incubated in duodenal and jejunal loops ($t_{1/2}$ = 0.4 min), while linaclotide injected in ileal loops was metabolized more slowly ($t_{1/2}$ = 36 min).

Metabolism of Linaclotide in Vitro in Intestinal Fluid. To further explore the metabolism of linaclotide in rodent intestine in vitro, linaclotide was incubated in a solution obtained from ligated intestinal loops from rats and concentrations of active peptide were determined by LC/MS/MS. A 30-min incubation resulted in the complete degradation of linaclotide. MM-419447, the active metabolite, formed almost immediately and reached a maximum concentration 10 min after incubation, after which the rate of degradation exceeded the rate of formation. Both peptides were degraded within 60 min. A related pattern of metabolism and degradation was observed when linaclotide was incubated in intestinal fluid from a mouse. Linaclotide is metabolized in a similar manner in intestinal fluid isolated from human cadavers, with immediate formation of MM-419447 and subsequent degradation of this metabolite. These experiments were carried out using intestinal fluids obtained from a particular region of

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the rodent or human intestine (proximal jejunum) using different preparation methods, and the results do not necessarily reflect the in vivo kinetics of linaclotide metabolism/degradation after oral administration. These experiments were conducted to study only the process of linaclotide metabolism in the intestine.

Pathway of Linaclotide Metabolism in Rat Intestinal Fluid. Reaction conditions were chosen to trap intermediates of the metabolism of linaclotide in rat intestinal fluid. After incubation of linaclotide in diluted intestinal fluid (10% loop fluid and 90% protein-filtered loop fluid), peptides detected included native linaclotide, native MM-419447, linaclotide with one reduced disulfide bond and two CAM-cysteines, and MM-419447 with one disulfide bond reduced and two CAM-cysteines. Similar results were obtained when [$^{13}C_1$]-Ala-linaclotide was incubated under the conditions described above. These results indicate that reduction of the disulfides bonds of linaclotide and MM-419447 is a necessary initial step in their degradation in intestinal fluid.

In a second experiment, proteolytic fragments of linaclotide and MM-419447 were trapped by slowing the reaction rate with diluted intestinal fluid, stopping the metabolism by removing proteins in the loop fluid using ultrafiltration, and reducing and alkylating the resulting sulfhydryl groups with iodoacetamide. Accurate mass measurements from LC-TOF/MS and MS/MS peptide sequencing was used to determine the identities of the fragments. All incubations were performed with linaclotide and [$^{13}C_1$]-Ala-linaclotide to confirm fragment identities. Figure 3 shows a list of the fragments of linaclotide that were detected in this experiment. The fragments are listed in three groups representing various digestion patterns (Figure 3). In these experiments, the digestion reactions were deliberately stopped at 15, 60 and 120 min in order to trap reduced forms and peptide

fragments. After overnight incubation, the digestion products were indistinguishable from the background species present in a reaction conducted without linaclotide (not shown). Figure S1 shows the early appearance and then disappearance of selected peptide fragments of linaclotide resulting from digestion in rat intestinal fluid as a function of time. Similar results were obtained when two fragments of linaclotide, MM-421202 (sequence CCEY) and MM-421522 (sequence CCNPACTGCY) were incubated overnight under the same conditions, resulting in their complete degradation (data not shown).

When an analogous experiment was conducted using a preparation of human intestinal fluid obtained from cadavers, a subset of the proteolytic fragments of linaclotide found in the rat experiment was observed (shown in Figure 3, bolded peptides), indicating a common linaclotide metabolism/degradation pathway in both rats and humans.

Analysis of the Pharmacological Activity of Reduced and Alkylated Linaclotide and Linaclotide Fragments. Reduced and alkylated linaclotide and MM-419447 did not elicit detectable cyclic GMP accumulation activity in T84 cells when tested at 1, 10, 100, and 1,000 nM (Figure S3A), while linaclotide and MM-419447 in their oxidized forms did (Figure S3A).

The peptides MM-421202 (CCEY) and MM-421522 (CCNPACTGCY) are two fragments detected after digestion of linaclotide in rat intestinal fluid (Figure 3) which together span the linear sequence of linaclotide. MM-421202 and MM-421522 were synthesized and tested for their ability to stimulate cGMP synthesis in T84 cells. MM-

421202 and MM-421522 did not induce cGMP accumulation activity in T84 cells when tested at 1, 100, and 1,000 nM, concentrations at which linaclotide is active (Figure S3B).

Complete Digestion of Linaclotide and MM-419447 to Naturally Occurring Amino Acids. In order to detect amino acids as products of linaclotide metabolism in rat intestinal fluid, the incubations were conducted for 23 hours at 37°C in reaction mixtures containing 10% concentrated ligated rat intestinal loop fluid, 90% PBS supplemented with 2 mM reduced glutathione, and 1 mM NADPH. Under these conditions, linaclotide, MM-42102, and MM-421522 were completely digested (data not shown). Table ST1 summarizes the concentrations of amino acids measured using UPLC after digestion of these peptides under the conditions described above and subsequent amino acid derivatization using the AccQ·Tag reagent. Overnight incubation resulted in complete digestion of the peptides, with quantitative recovery of internal amino acids. In this experiment, twice as much tyrosine was recovered from linaclotide as from MM-419447, as expected from the sequences. Also, there was no glutamic acid detected after complete digestion of MM-421522 since this peptide lacks this amino acid residue. Based on the amount of linaclotide incubated, UPLC analysis demonstrated nearly quantitative recovery of the tyrosine, alanine, proline, and asparagine amino acids, as well as most of the threonine in the linaclotide sample.

Linaclotide and MM-419447 Binding to Intestinal Guanylate Cyclase C Receptors. We have previously shown that GC-C is the molecular target of linaclotide (Bryant et al. 2010), and that linaclotide binds to T84 cells, which express high levels of guanylate cyclase C, in a concentration-dependent and pH-independent manner (Busby et al., 2010). To compare the pharmacological activity of MM-419447 to that of linaclotide,

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synthetic MM-419447 was tested for its affinity to cell surface receptors in T84 cells. When MM-419447 was tested in a competitive radioligand-binding assay with [^{125}I]-pSTa, a known agonist of GC-C, the binding of MM-419447 to cell-surface receptors on T84 cells was very similar to that of linaclotide at pH 7 (Figure S2 and Table 4). Likewise, MM-419447 binds cell-surface receptors in rat small intestine brush border membranes (pH 7) with a relative affinity comparable to linaclotide (Table 4). The binding of MM-419447 to receptors on T84 cells at pH 5 and pH 8 is also comparable to that of linaclotide, indicating that the binding affinities of linaclotide and its metabolite are pH independent. Comparison of the competitive binding curves of linaclotide and MM-419447 using the F test indicated that their IC_{50} and K_i values were not statistically different ($P > 0.05$).

Effect of Linaclotide and MM-419447 on cGMP Accumulation in Human T84 Cells. Binding of linaclotide to GC-C receptors stimulates the intrinsic guanylate cyclase activity of the receptor, resulting in increased concentrations of intracellular cGMP (Busby et al., 2010). MM-419447 also stimulates the accumulation of cGMP in human T84 cells in a concentration-dependent manner with statistically equivalent potency. The concentration-response curves were used to calculate the EC_{50} values for linaclotide and MM-419447, 112 ± 21.1 nM and 103 ± 26 nM, respectively. Comparison of the concentration-response curves of linaclotide and MM-419447 using the F test indicated that the EC_{50} values were not statistically different ($P = 0.7555$). These results indicate that MM-419447 acts as an agonist of guanylate cyclase C in vitro, with potency similar to that of linaclotide.

Effect of MM-419447 on Fluid Secretion and cGMP Accumulation in Rat Small Intestinal Loops. We further investigated whether the in vitro pharmacological

properties of MM-419447 would translate in vivo into common rat models of gastrointestinal function. The stimulation of intestinal fluid secretion was assessed by measuring the volume of the luminal fluid content 90 min after the exposure of ligated duodenal, jejunal, and ileal loops to either 5 µg of MM-419447 or vehicle. Treatment with MM-419447 stimulated a significant increase in fluid secretion compared to vehicle-treated animals in loops from all regions of the small intestine (Figure 4A). This increase in fluid secretion after treatment with MM-419447 was accompanied by a marked increase in the intra-luminal concentration of cGMP in the duodenum and jejunum, and a much less pronounced increase in cGMP in ileal loops (Figure 4B). Similar results for increased fluid secretion and concomitant cGMP secretion in the duodenum and jejunum were observed previously after treatment with linaclotide (Busby et al., 2010). Low concentrations of cGMP accumulation in ileal loops injected with STa have been reported by others (London et al., 1997; Qian et al., 2000).

Effects of Linaclotide and MM-419447 on Gastrointestinal Transit in Female Rats. We measured the effects of orally administered MM-419447 and linaclotide in a dose-dependent manner (6.25 µg/kg to 50 µg/kg) in a rat gastrointestinal transit model. Gastrointestinal transit was determined by measuring the distance a dose of activated charcoal, administered orally immediately after MM-419447 or linaclotide dosing, traveled in the small intestine after 10 min relative to the total length of the small intestine. Oral administration of MM-419447 or linaclotide resulted in a significant and dose-dependent acceleration of gastrointestinal transit, compared to vehicle-treated rats (except for the 6.25 µg/kg MM-419447 dose group). The traveled distances ranged from 57.8% to 66.4% and 59.1% to 65% for MM-419447 and linaclotide, respectively,

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compared to 50.3% in vehicle-treated rats (Figure 5). A side-by-side comparison of each dose of MM-419447 and linaclotide using two-way ANOVA revealed that their dose-response curves were not significantly different ($P = 0.6876$), suggesting equal potency in this model. Linaclotide also significantly accelerated gastrointestinal transit in male CD rats (data not shown).

Discussion

GC-C is a receptor for the endogenous peptide hormones guanylin and uroguanylin and is predominantly expressed on the apical surface of epithelial cells of the small intestine and colon (Krause et al., 1994). Linaclotide is an orally administered 14-amino acid peptide agonist of GC-C which acts locally within the lumen of the GI tract to exert its effects without a need for systemic exposure

The amino acid sequence and tertiary molecular structure of linaclotide differ from those of guanylin and uroguanylin. These hormones contain four cysteines, as opposed to the six within linaclotide. These extra cysteines allow linaclotide to form an additional intramolecular disulfide bond. This third disulfide bond enhances the relative GC-C agonist potency of linaclotide by locking it into its active conformation, strengthening its binding affinity for GC-C and increasing its stability within the GI tract. However, the reinforced tertiary structure of linaclotide does not prevent the proteolytic removal of its C-terminal tyrosine, which is not protected by the disulfide bonds, resulting in the formation of a 13-amino acid metabolite, MM-419447 (Des-Tyr^{14}), described here for the first time.

Linaclotide is resistant to the harsh, acidic conditions of the stomach, as shown by its stability in simulated gastric fluid. Under non-reducing conditions it is also resistant to proteolysis by gastrointestinal hydrolases, with the exception of carboxypeptidase A. This enzyme removes the C-terminal Tyr^{14} residue of the parent peptide, leaving the three intramolecular disulfide bonds and the tertiary structure necessary for increased protease resistance, GC-C binding, and potent GC-C agonist activity intact. The resulting peptide has been confirmed as a circulating metabolite in high-dose pharmacokinetic experiments

performed in mice, rats, dogs, and monkeys (data not shown). The detection of MM-419447 in stool samples from human subjects orally dosed with linaclotide confirms that MM-419447 is also a human metabolite, although it hasn't been found in systemic circulation.

To determine whether this metabolite contributes to the pharmacological effects of linaclotide, we synthesized MM-419447, characterized its in vitro pharmacological properties, and tested it in rat models of gastrointestinal function. MM-419447 exhibited in vitro binding to T84 cell surface receptors similar to that of linaclotide. In addition, MM-419447 significantly increased intracellular cGMP concentrations in these cells in a concentration-dependent manner, with similar potency to that of linaclotide. These results are consistent with the hypothesis that MM-419447, like linaclotide, is a potent agonist of GC-C and provide further evidence that MM-419447 contributes to the in vivo pharmacological effects observed with linaclotide.

The ability of MM-419447 to stimulate fluid secretion was measured in vivo following injection into surgically ligated duodenal, jejunal, and ileal loops in rats. Fluid volume was significantly increased in all three small intestinal regions. This increased fluid secretion was associated with increased luminal cGMP concentrations, suggesting that MM-419447 acts through stimulation of GC-C receptors. The same pattern and magnitudes of biological responses were observed with linaclotide (Busby et al., 2010). The pharmacological effects of orally administered MM-419447 on the rate of gastrointestinal transit in rats were also assessed, as altered intestinal transit correlates with clinical symptoms of IBS-C and chronic constipation (Mayer et al., 2008). MM-419447 showed a significant and dose-dependent acceleration in the rate of

gastrointestinal transit in rats that was similar to that seen for linaclotide, further supporting the hypothesis that MM-419447 is a potent agonist in vivo of GC-C and contributes to the local pharmacology of linaclotide within the intestine.

Recent evidence suggests a role for cGMP in mediating linaclotide's effects on visceral pain (Castro et al., 2012, submitted for publication). Like linaclotide, MM-419447 stimulates cGMP production in the intestinal epithelium, and therefore this active metabolite may contribute to the analgesic effects observed after oral administration of linaclotide.

The disposition of linaclotide and MM-419447 was also assessed. Pharmacokinetic studies in rats using orally and intravenously dosed MM-419447 demonstrated that, as with linaclotide, MM-419447 is minimally absorbed, and has an absolute oral bioavailability of $\leq 0.1\%$ in rats. Following a 10 mg/kg oral dose of linaclotide, jugular and portal vein plasma concentrations of both linaclotide and MM-419447 were low (≤ 6 ng/ml) in rats, indicating that both peptides are poorly absorbed and hepatic exposure to them is minimal. Minimal systemic exposure to linaclotide was also observed in humans in three Phase 1 studies and was confirmed with sparse PK sampling in four Phase 3 trials. Plasma concentrations of linaclotide were typically not measurable and were always below 1 ng/ml. MM-419447 was never quantifiable (LLOQ = 2 ng/ml) and was therefore not confirmed as a circulating metabolite in humans, although it was observed in human stool samples. The minimal systemic availability of linaclotide in humans is consistent with its low permeability coefficient in Caco-2 cells (data not shown) and the low absolute oral bioavailability found in animals.

Despite the fact that both active peptides are minimally absorbed, the majority of dosed linaclotide is not excreted in the feces. In rats, less than 1% of the orally delivered drug was recovered in feces as linaclotide or MM-419447. Of the recovered peptide, > 95% was in the form of the active metabolite MM-419447. This confirms that the vast majority of the drug is broken down within the intestine prior to excretion. Moreover, the results of a Phase 1 study in humans showed that a mean of 3-5% of the oral linaclotide dose was excreted in the feces as pharmacologically active peptide. As was observed in rats, MM-419447 was the predominant peptide detected. Nevertheless, the presence of parent and active metabolite in the feces demonstrates that some active peptide is available to stimulate the GC-C receptor throughout the small intestine and colon.

Since linaclotide is minimally absorbed into systemic circulation and the majority of the drug is not excreted either as parent or active metabolite in the feces, the intestinal fate of linaclotide is proteolytic digestion. Using a series of in vitro and in vivo experiments in rodents, as well as in human biomaterials from cadavers, we have characterized this degradation pathway. We observed rapid proteolysis of linaclotide in the duodenum and jejunum of the rat and a slower rate of disappearance in the distal small intestine (ileum). The proteolytic metabolism was confirmed with subsequent in vitro studies in which linaclotide was incubated with intestinal fluid from mice, rats, and humans. In these studies, linaclotide decreased immediately while MM-419447 began to form immediately.

The digestion of either linaclotide or MM-419447 requires a process that first reduces their disulfide bonds. The intestine is known to have the capacity to reduce disulfide bonds in proteins and peptides (Dahm and Jones, 2000; Fernandes and Holmgren, 2004;

Avval and Holmgren, 2009). Similar to our previous observations in rats, the luminal contents of the human intestine were found to contain high thiol concentrations (3-8 mM) and the components of the glutaredoxin system, which has been shown to reduce the three disulfide bonds of linaclotide (unpublished). Disulfide bond reduction leaves both linaclotide and MM-419447 highly susceptible to proteolytic degradation, as demonstrated by the rapid in vitro cleavage of reduced linaclotide by proteolytic enzymes, including chymotrypsin. The degradation pathway of linaclotide was further characterized by incubating it with diluted rat intestinal fluid to slow down the digestion rate and allow intermediate peptide degradation products to be trapped and identified. These linearized peptide fragments contained three or more amino acids. Some of these peptide fragments were synthesized and were found to have no GC-C agonist activity. Overnight digestion of the synthesized fragments resulted in their complete degradation, with quantitative recovery of internal amino acids. Isolation and identification of similar peptide intermediates following human intestinal fluid incubation when compared to those from rat intestinal fluid incubations suggest that the metabolism and degradation pathway of linaclotide in humans is the same as that in the rat. Figure 3 shows the intestinal fate of linaclotide, beginning with its conversion to MM-419447, followed by disulfide bond reduction, which inactivates both peptides. Intestinal hydrolases are then able to proteolytically degrade the linearized peptides to smaller peptides and amino acids, which are subsequently recycled. Individual amino acids are absorbed through the intestine, as are peptides consisting of two or three amino acids (Daniel 2004; Silk et al., 2004), while hexa-, penta-, and tetrapeptides are further digested by brush border proteases prior to absorption (Kim et al., 1972).

In conclusion, a more complete understanding of the pharmacology and drug disposition of linaclotide now exists. Linaclotide is readily metabolized to an active metabolite, MM-419447, which mirrors the parent in both pharmacological and pharmacokinetic properties. Both peptides act locally in the intestinal lumen with minimal absorption into systemic circulation. Under the conditions found in the intestine, both active peptides are broken down in a process that requires disulfide bond reduction, which enables proteolytic digestion and results in a loss of pharmacological activity. Orally administered linaclotide is confined primarily to the intestine, the site of both pharmacological activity and metabolic clearance, thereby allowing linaclotide and its active metabolite to exert their pharmacologic effects while minimizing systemic exposure to the peptides.

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References

Andresen V, Camilleri M, Busciglio IA, Grudell A, Burton D, McKinzie S, Foxx-Orenstein A, Kurtz CB, Sharma V, Johnston JM, Currie MG, and Zinsmeister AR (2007) Effect of 5 days linaclotide on transit and bowel function in females with constipation-predominant irritable bowel syndrome. *Gastroenterology* **133:**761-768.

Avval FZ and Holmgren A (2009) Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for mammalian S phase ribonucleotide reductase. *J Biol Chem* **284:**8233-8240.

Bauer CW and Miskinis AM (1947) The carboxypeptidase activity of pancreatin U.S.P. *J Am Pharm Assoc***36:**123-126

Brandwein, H., Deutsch A., Thompson M., and Giannella R.A. (1985) Production of neutralizing monoclonal antibodies to *Escherichia coli* heat-stable enterotoxin**.** *Infect Immun* **47:** 242-46.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal Biochem* **72:**248-254.

Bryant AP, Busby RW, Bartolini WP, Cordero EA, Hannig G, Kessler MM, Pierce CM, Solinga RM, Tobin JV, Mahajan-Miklos S, Cohen MB, Kurtz CB and Currie MG (2010) Linaclotide is a potent and selective guanylate cyclase C agonist that elicits pharmacological effects locally in the gastrointestinal tract. *Life Sci* **86:**760-765.

Busby RW, Bryant AP, Bartolini WP, Cordero EA, Hannig G, Kessler MM, Mahajan-Miklos S, Pierce CM, Solinga RM, Sun LJ, Tobin JV, Kurtz CB and Currie MG (2010)

Linaclotide, through activation of guanylate cyclase C, acts locally in the gastrointestinal tract to elicit enhanced intestinal secretion and transit. *Eur J Pharmacol* **649:**328-335.

Chey WD, Lembo AJ, Lavins, BJ, Shiff, SJ, Kurtz, CB, Currie, MG, MacDougall, JE, Jia, XD, Shao JZ, Fitch DA, Baird MJ, Schneier HA, and Johnston JM (2012) Linaclotide for Irritable Bowel Syndrome with Constipation: A 26-week, Randomized, Double-blind, Placebo-controlled Trial to Evaluate Efficacy and Safety. *Am J Gastroenterol* doi: 10.1038/ajg.2012.254.

Cohen MB, Giannella RA (1992) Jejunal toxin inactivation regulates susceptibility of the immature rat to STa. *Gastroenterology* **102:**1988-96.

Cohen MB, Moyer MS, Luttrell M, and Giannella RA (1986) The immature rat small intestine exhibits an increased sensitivity and response to Escherichia coli heat-stable enterotoxin. *Pediatr Res* **20:**555-560.

Currie MG, Fok KF, Kato J, Moore RJ, Hamra FK, Duffin KL and Smith CE (1992) Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proc Natl Acad Sci U S A* **89:**947-951.

Dahm LJ and Jones DP (2000) Rat jejunum controls luminal thiol-disulfide redox. *J Nutr* **130:**2739-2745.

Daniel H. (2004) Molecular and integrative physiology of intestinal peptide transport. *Ann Rev Physiol* **66:**361-84.

Eutamene H, Bradesi S, Larauche M, Theodorou V, Beaufrand C, Ohning G, Fioramonti J, Cohen M, Bryant AP, Kurtz C, Currie MG, Mayer EA and Bueno L (2010) Guanylate cyclase C-mediated antinociceptive effects of linaclotide in rodent models of visceral pain. *Neurogastroenterol Motil* **22:**312-322.

Fan X, Hamra FK, Freeman RH, Eber SL, Krause WJ, Lim RW, Pace VM, Currie MG, Forte LR (1996). Uroguanylin: cloning of preprouroguanylin cDNA, mRNA expression in the intestine and heart and isolation of uroguanylin and prouroguanylin from plasma. *Biochem Biophys Res Commun* **219:**457-462.

Fernandes AP and Holmgren A. (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antiox & Redox Signal* **6:**63-74.

Fondacaro JD, Kolpak DC, Burnham DB, McCafferty GP (1990) Cecectomized rat: a model of experimental secretory diarrhea in conscious animals. *J Pharmacol Meth* **24:**59-71.

Forte LR (2004) Uroguanylin and guanylin peptides: pharmacology and experimental therapeutics. *Pharmacol Ther* **104:**137-162.

Hamra FK, Forte LR, Eber SL, Pidhorodeckyj NV, Krause WJ, Freeman RH, Chin DT, Tompkins JA, Fok KF, Smith CE, Duffin, KL, Siegel ND and Currie MG (1993) Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. *Proc Natl Acad Sci U S A* **90**: 10464-10468.

Hamra FK, Fan X, Krause WJ, Freeman RH, Chin DT, Smith CE, Currie MG, Forte LR(1996) Prouroguanylin and proguanylin: purification from colon, structure, and modulation of bioactivity by proteases. *Endocrinol* **137:**257-265.

Hamra FK, Eber SL, Chin DT, Currie MG, Forte LR (1997) Regulation of intestinal uroguanylin/guanylin receptor-mediated responses by mucosal acidity. *Proc Natl Acad Sci USA* **94:**2705-2710.

Johnson LR, Alpers DH, Christensen J, Jacobson ED, editors. Physiology of the gastrointestinal tract, New York: Raven Press; 1994.

Johnston JM, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, O'Dea C, Baird M, and Lembo AJ (2010) Linaclotide improves abdominal pain and bowel habits in a phase IIb study of patients with irritable bowel syndrome with constipation. *Gastroenterology* **139:**1877-1886.

Kessler JI, Stein J, Dannacker D and Narcessian P (1970) Biosynthesis of low density lipoprotein by cell-free preparations of rat intestinal mucosa. *J Biol Chem* **245:**5281-5288.

Lembo AJ, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, Jeglinski BI, Johnston JM (2010) Efficacy of linaclotide for patients with chronic constipation. *Gastroenterology* **138:**886-895.

Lembo AJ, Schneier HA, Shiff SJ, Kurtz CB, MacDougall JE, Jia XD, Shao JZ, Lavins BJ, Currie MG, Fitch DA, Jeglinski BI, Eng P, Fox SM and Johnston JM (2011) Two randomized trials of linaclotide for chronic constipation. *N Engl J Med* **365**:527-536.

London RM, Krause WJ, Fan X, Eber SL and Forte LR (1997) Signal transduction pathways via guanylin and uroguanylin in stomach and intestine. *Am J Physiol* **273:**G93-G105.

Marques M **(**2004) Dissolution Media Simulating Fasted and Fed States. *Dissolution Technologies* **11:**16.

Mayer EA, Bradesi S, Chang L, Spiegel BMR, Bueller JA, Naliboff BD (2008) Functional GI disorders: from animal models to drug development. *Gut* **57:**384-404.

QianX, Prabhakar S, Nandi A, Visweswariah SS, and Goy, MF, (2000) Expression of GC- C, a receptor-guanylate cyclase, and its endogenous ligands uroguanylin and guanylin along the rostrocaudal axis of the intestine. *Endocrinol* **141:**3210–3224.

Rao, S, Lembo, AJ, ; Shiff, SJ, Lavins BJ, Currie, MG, Jia, XD, Shi, K. MacDougall, JE, Shao, JZ, Eng, P, Fox, SM, Schneier, HA, Kurtz, CB, Johnston, JM. (2012) A 12-Week, Randomized, Controlled Trial with a 4-Week Randomized Withdrawal Period to Evaluate the Efficacy and Safety of Linaclotide in Irritable Bowel Syndrome with Constipation. *Am J Gastroenterol* doi: 10.1038/ajg.2012.2.

Roque MVand Camilleri M (2011) Linaclotide, a synthetic guanylate cyclase C agonist, for the treatment of functional gastrointestinal disorders associated with constipation. *Expert Rev Gastroenterol Hepatol* **5:**301-310.

Sanderink GJ, Artur Y, Siest G (1988) Human aminopeptidases: a review of the literature. *J Clin Chem Clin Biochem* **26:**795-807.

Schulz S, Green CK, Yuen PST, Garbers DL (1990) Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* **63:**941-948.

Thiagarajah JR, Broadbent T, Hsieh E, Verkman K (2004) Prevention of toxin-induced intestinal ion and fluid secretion by a small-molecule CFTR inhibitor. *Gastroenterology* **126:**511-19.

Thompson MR, Luttrell M, Overmann G, Giannella RA (1985) Biological and immunological characteristics of ^{125}I-4Tyr and -18Tyr *Escherichia coli* heat-stable enterotoxin species purified by high-performance liquid chromatography. *Anal Biochem* **148:**26-36.

Wensel TM and Luthin DR (2011) Linaclotide: a novel approach to the treatment of irritable bowel syndrome. *Ann Pharmacother* **45**:1535-1543.

Wiegand RC, Kato J, Currie MG (1992) Rat guanylin cDNA: characterization of the precursor of an endogenous activator of intestinal guanylate cyclase. *Biochem Biophys Res Commun* **185:**812-817.

Wilkinson GR (1997) The effects of diet, aging and disease-states on presystemic elimination and oral drug bioavailability in humans. *Adv Drug Del Rev* **27:** 129-159.

Woodley JF (1994) Enzymatic barriers for GI peptide and protein delivery. *Crit Rev Ther Drug Carrier Syst* **11:**61-95.

Yu Y, Jawa A, Pan W, Kastin AJ (2004) Effects of peptides, with emphasis on feeding, pain and behavior. A 5-year (1999-2003) review of publications in peptides. *Peptides* **25**:2257-2289.

Footnotes

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Legends for Figures

Fig. 1. Stability of linaclotide in the presence of carboxypeptidase A. Linaclotide was incubated in the presence of carboxypeptidase A under non-reducing conditions for up to 6 hours. Linaclotide and MM-419447 were monitored in these reactions by LC/MS. The values shown represent the mean ± standard deviation (*n*=3 per time point).

Fig. 2. Systemic (A) and portal vein (B) concentrations of linaclotide and MM-419447 as a function of time following oral administration of linaclotide. Linaclotide was administered by oral gavage (10 mg/kg) and the plasma concentrations of linaclotide and MM-419447 were determined at specific time intervals by LC/MS/MS. The quantitation limit of linaclotide and MM-419447 in rat plasma was 1 ng/ml and 2 ng/ml, respectively. The values shown represent the mean ± S.E.M (*n*=4 per time point).

Fig. 3.Trapping and identification of linaclotide digestion intermediates after incubation in rat intestinal loop fluid. Linaclotide was incubated in diluted intestinal fluid for 15, 60, and 120 min, and overnight. At each time point, proteins were removed and the disulfide bonds in peptides were reduced with DTT followed by alkylation with iodoacetamide. The resulting peptides were identified by LC-TOFMS. Bolded peptides were detected in both rat and human intestinal fluids after incubation of linaclotide.

Fig. 4. MM-419447 stimulates fluid secretion and cGMP accumulation in female rat intestinal loops. (A) Ligated loops were surgically introduced into the duodenum, jejunum, and ileum, and either MM-419447 (5 μg), linaclotide (5 μg), or vehicle was

injected into these loops. After 90 min, the rats were sacrificed, the loops were excised, and the weight-to-length (W/L) ratio was determined. All data are expressed as the mean ± S.E.M (n = 10). ** = $P < 0.01$, *** = $P < 0.001$ versus vehicle. (B) The concentration of cGMP in luminal fluid recovered from the loops was measured using a competitive enzyme immunoassay. All data are expressed as the mean ± S.E.M (n=10). * = $P < 0.05$ versus vehicle.

Fig. 5. MM-419447 accelerates gastrointestinal transit in female rats. Rats were administered either vehicle or increasing doses of MM-419447 or linaclotide by oral gavage, immediately prior to an oral dose of 10% activated carbon/10% gum arabic powder suspension. After 10 min, the rats were sacrificed and gastrointestinal transit was determined as the percentage of the distance traveled by the charcoal front compared to the total length of the intestine. All data are expressed as the mean ± S.E.M (n=5-10 per group). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ versus vehicle.

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Tables

TABLE 1

Pharmacokinetic parameters following oral and intravenous administration of MM-419447 to rats (10 mg/kg)

Parameter	MM-419447
T_{max} (h)	0.33
C_{max}(ng/ml)	27.0
$AUC_{i.v.(0\text{-}6h)}$ (ng-h/ml)[a]	36,100
$AUC_{p.o.(0\text{-}6h)}$ (ng-h/ml)	$\leq$ 29.7[a]
Bioavailability (%)	$\leq$ 0.1[a]

[a.] The $\leq$ symbol indicates that the reported value is an upper estimate of the AUC because the concentration of the lowest standard was used as the concentration of any sample with a concentration lower that the quantitation limit.

TABLE 2

Summary of Pharmacokinetic results from clinical studies* performed with linaclotide

Study Number	**Linaclotide Doses (Number of PK Subjects)**	**Linaclotide Plasma Concentrations**	**MM-419447 Plasma Concentrations**
PK STUDIES IN HEALTHY VOLUNTEERS			
MCP-103-001 (Phase 1, US)	Single 29 µg (4); 97 µg (8); 290 µg (4); 966 µg (4); 2897 µg (4)	Not detectable (< 3.00 ng/ml)	Not detectable (< 3.00 ng/ml)
0456-CL-0011 (Phase 1, Japan)	Single 109 µg (6); 327 µg (6); 1090 µg (6); 3,270 µg (6)	Not detectable (< 0.200 ng/ml)	Not detectable (< 2.00 ng/ml)
MCP-103-002 (Phase 1, US)	QD x 7 days 29 µg (8); 97 µg (8); 290 µg (8); 966 µg(8)	Not detectable (< 3.00 ng/ml)	Not detectable (< 3.00 ng/ml)
MCP-103-103 (Phase 1 food effect, US)	QD x 7 days 290 µg (9 Fed, 10 Fasted)	Not detectable (< 0.200 ng/ml)	Not detectable (< 2.00 ng/ml)
	Single[1] 2,897 µg (9 Fed, 9 Fasted)	Not detectable (< 0.200 ng/ml) except in 2 fasted subjects: Subject 12 C_{max} = 0.735 ng/ml Subject 19 C_{max} = 0.212 ng/ml	Not detectable (< 2.00 ng/ml)

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SPARSE SAMPLING (DAY 1 AND 29) IN PATIENTS WITH CC			
LIN-MD-01 (Phase 3)	QD x 12 weeks 145 µg (51); 290 µg (53)	Not detectable (< 0.200 ng/ml)	Not detectable (< 2.00 ng/ml)
MCP-103-303 (Phase 3)	QD x 12 weeks 145 µg (101); 290 µg (98)	Not detectable (< 0.200 ng/ml)	Not detectable (< 2.00 ng/ml)
SPARSE SAMPLING (DAY 1 AND 29) IN PATIENTS WITH IBS-C			
LIN-MD-31 (Phase 3)	QD x 12 weeks 290 µg (64)	Not detectable (< 0.200 ng/ml)	Not detectable (< 2.00 ng/ml)
MCP-103-302 (Phase 3)	QD x 26 weeks 290 µg (98)	Not detectable (< 0.200 ng/ml) except in 2 patients on Day 1 Patient 007-2006 = 0.241 ng/ml Patient 049-2006 = 0.239 ng/ml	Not detectable (< 2.00 ng/ml)

* The clinical protocols and all clinical trial procedures were approved by an Institutional Review Board, and the trial was designed, conducted, and reported in accordance with the principles of Good Clinical Practice guidelines. All patients gave written informed consent before their participation in the trial.

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TABLE 3

Recovery of linaclotide and MM-419447 from human feces after oral administration of linaclotide. Values are shown as percent of oral dose.

Fasted		Fasted		Fed		Fed	
Dose 290 µg		Dose 2,897 µg		Dose 290 µg		Dose 2,897 µg	
Subject	%Active Peptide†	Subject	%Active Peptide†	Subject	%Active Peptide†	Subject	%Active Peptide†
002	5.97	002	0*	001	NS	001	2.21
004	0.39	004	0.28	003	2.11	003	3.72
005	2.53	005	2.94	006	0*	006	0*
008	NS	008	0*	007	0.75	007	1.06 [1]
011	NS	011	0*	009	8.18	009	5.20
012	0.49	012	5.73	010	2.51	010	2.57 [2]
015	0.37	015	2.78	016	9.37	016	20.84 [3]
018	6.72	018	11.76	017	0*	017	0*
019	17.75	019	6.57	020	1.97	020	0*
Median	2.53	Median	2.78	Median	2.04	Median	2.21
Mean	4.89	Mean	3.34	Mean	3.11	Mean	3.96
SD	6.3	SD	4.0	SD	3.6	SD	6.6

SD, standard deviation
NS, no sample available
† % Active peptide present as MM-419447 unless noted
* below detection limits
[1] 0.88% MM-419447, 0.18% linaclotide
[2] 2.22% MM419447, 0.35% linaclotide
[3] 19.97% MM-419447, 0.87% linaclotide

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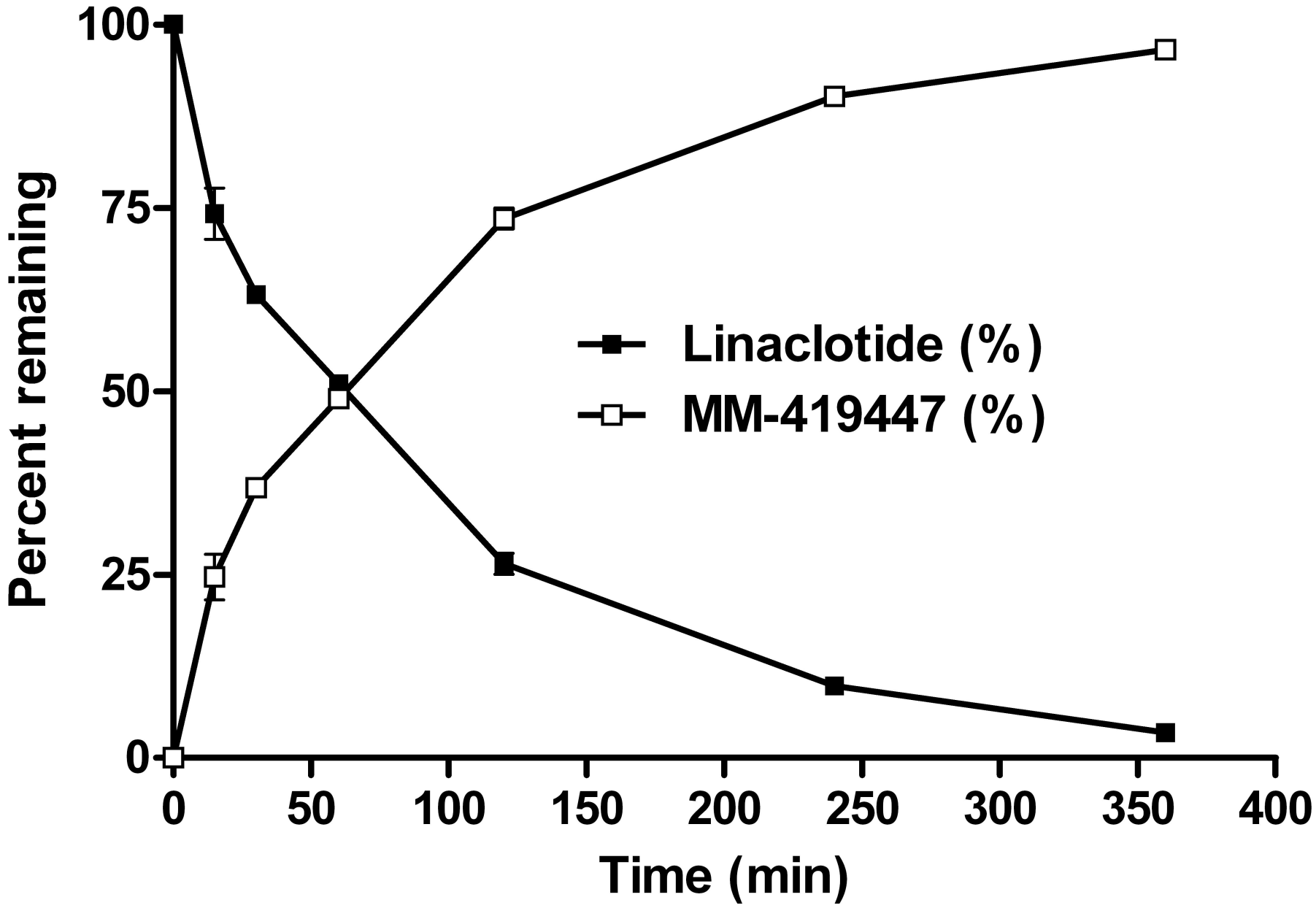
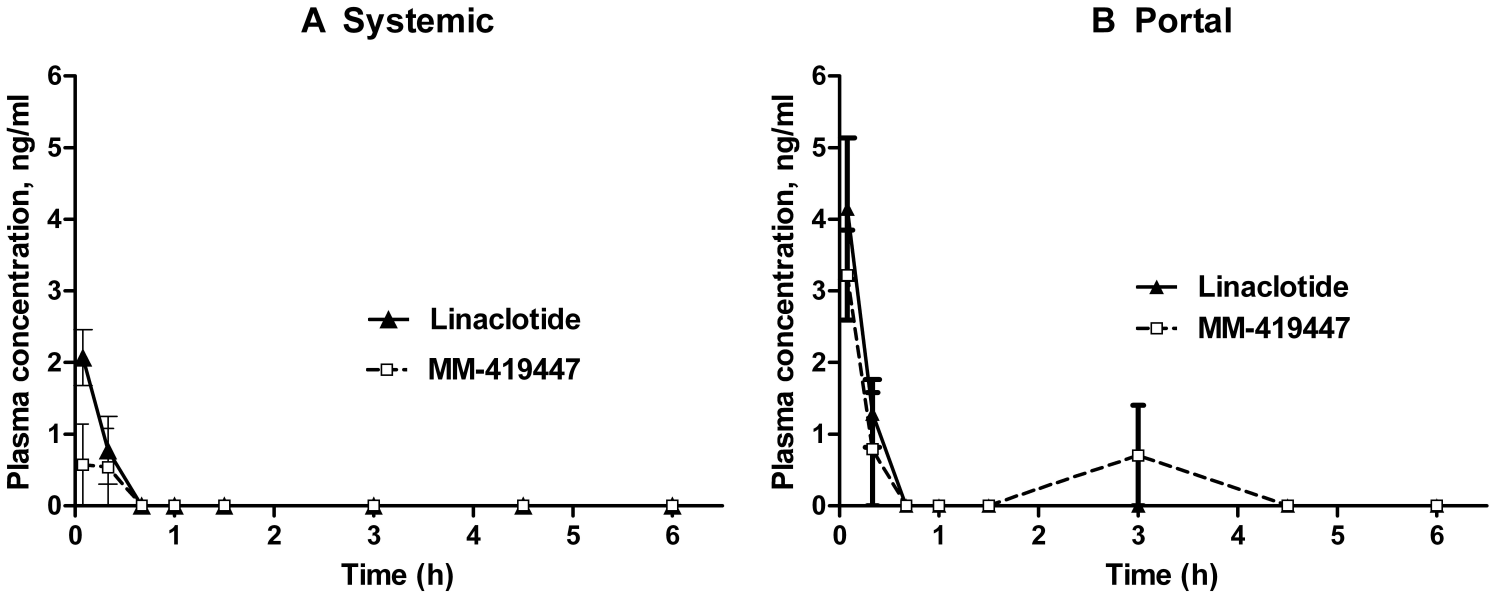
TABLE 4

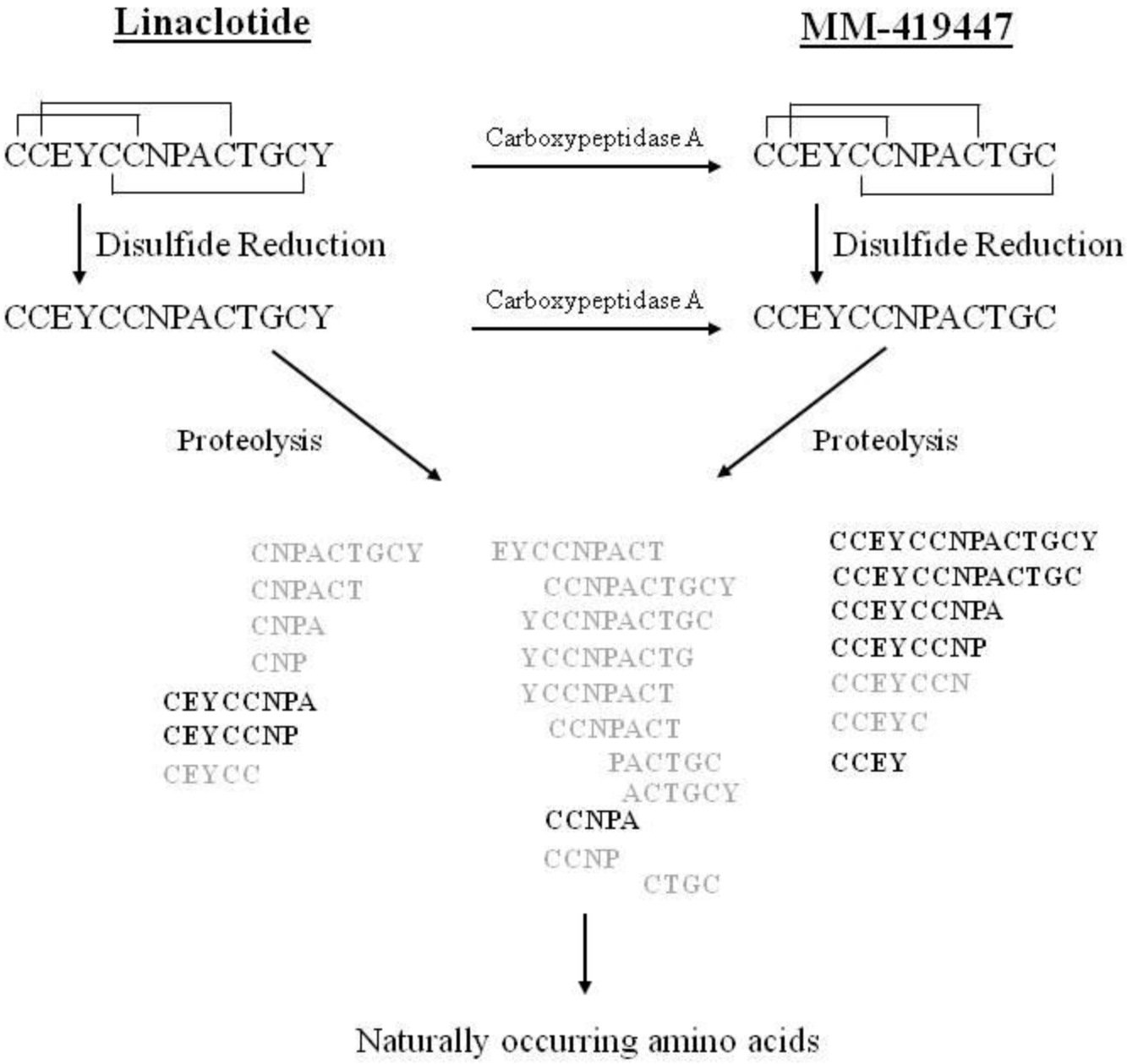
Summary of the relative binding affinities of linaclotide and MM-419447

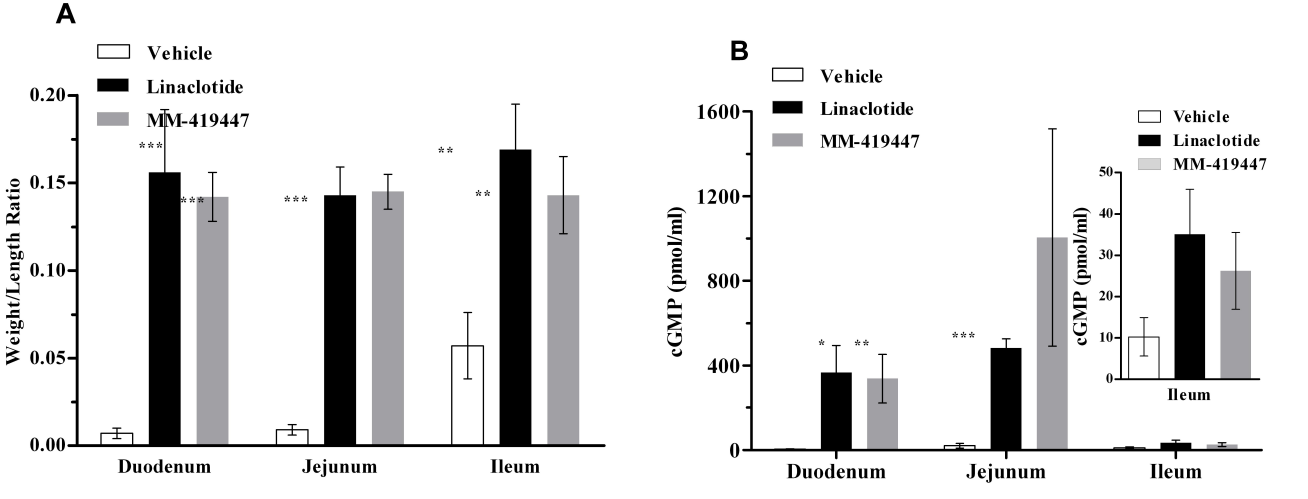
Binding assay	K_i ± standard error (nM)	
	Linaclotide	MM-419447
T84 cells, pH 5	1.7 ± 0.8	0.9 ± 0.5
T84 cells, pH 7	3.1 ± 1.4	1.8 ± 1.0
T84 cells, pH 8	1.5 ± 1.8	1.6 ± 1.2
Rat intestinal BBM, pH 7	2.6 ± 2.9	4.9 ± 2.8

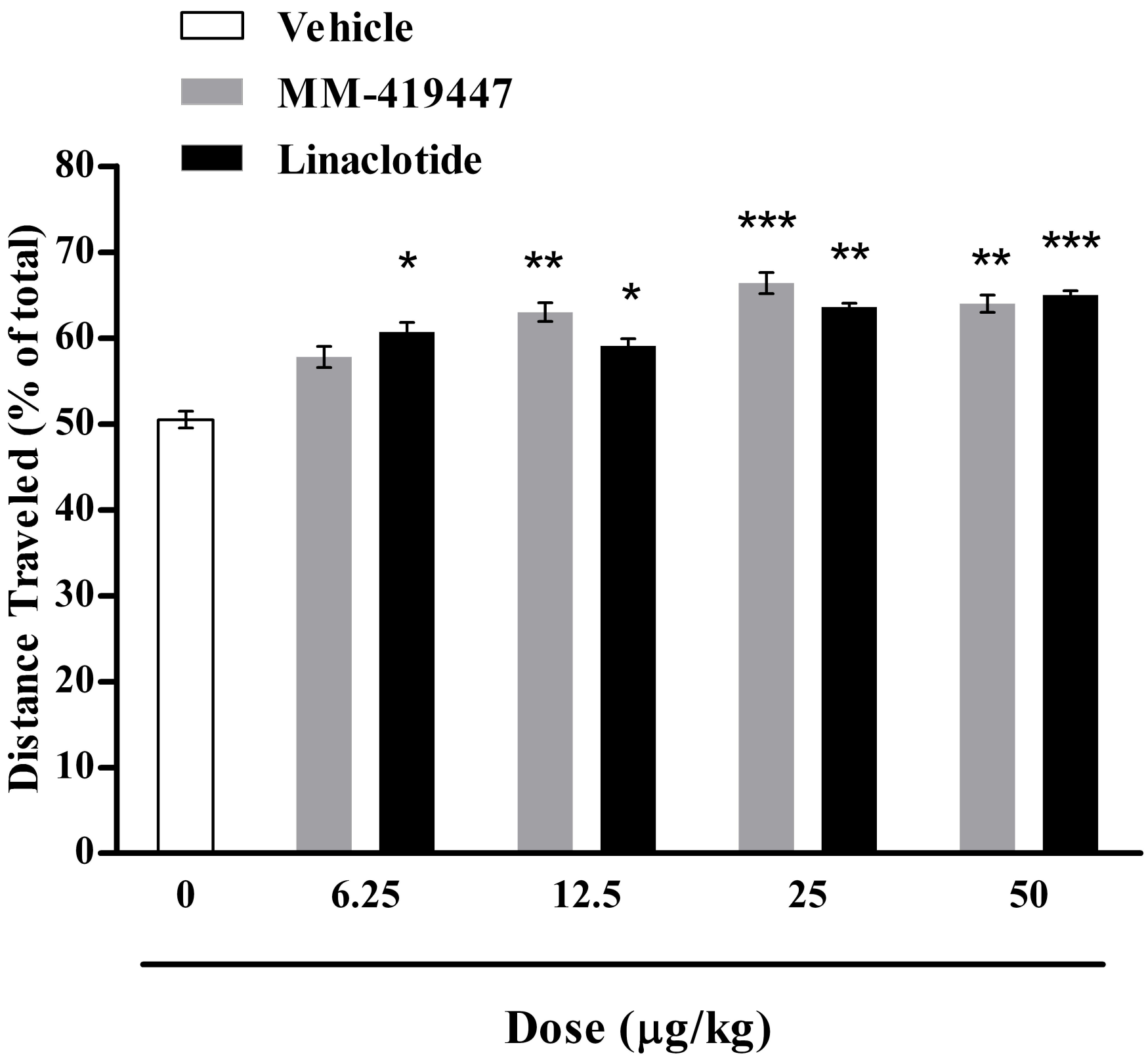
BBM, brush border membranes


100
75
50
25
0
Percent remaining
Linaclotide (%)
MM-419447 (%)
0
50
100
150
200
250
300
350
400
Time (min)


A Systemic
Plasma concentration, ng/ml
Time (h)
Linaclotide
MM-419447
B Portal
Plasma concentration, ng/ml
Time (h)
Linaclotide
MM-419447




A
Vehicle
Linaclotide
MM-419447
Weight/Length Ratio
0.20
0.15
0.10
0.05
0.00
***
***
***
**
**
Duodenum
Jejunum
Ileum
B
Vehicle
Linaclotide
MM-419447
cGMP (pmol/ml)
1600
1200
800
400
0
*
**
***
Duodenum
Jejunum
Ileum
Vehicle
Linaclotide
MM-419447
cGMP (pmol/ml)
50
40
30
20
10
0
Ileum


Vehicle
MM-419447
Linaclotide
Distance Traveled (% of total)
80
70
60
50
40
30
20
10
0
*
**
*
***
**
**
***
0
6.25
12.5
25
50
Dose (µg/kg)

SUPPLEMENTAL DATA

Journal of Pharmacology and Experimental Therapeutics

Pharmacological Properties, Metabolism and Disposition of Linaclotide, a Novel Therapeutic Peptide Approved for the Treatment of Irritable Bowel Syndrome with Constipation and Chronic Idiopathic Constipation[#]

Robert W. Busby*, Marco M. Kessler, Wilmin P. Bartolini, Alexander P. Bryant, Gerhard Hannig, Carolyn S. Higgins, Robert M. Solinga, Jenny V. Tobin, James D. Wakefield, Caroline B. Kurtz, and Mark G. Currie

Ironwood Pharmaceuticals, Inc.

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Contents:

Supplemental Methods

Stability of Linaclotide in Simulated Gastric Fluid (SGF)..

Triplicate reactions (0.5 ml) were assembled containing 0.1 mg/ml linaclotide or MM-419447 in SGF (0.2 M HCl, 34 mM NaCl, pH 1.0) or in100 mM Tris-HCl, pH 7.5 and incubated at 37°C. Aliquots were removed from each reaction at t_0 ("0 h"), 1 h, and 3 h and immediately added to ice-cold deionized water to dilute the SGF and analyzed using high-pressure liquid chromatography/high resolution mass spectrometry (LC/FTMS)using a Hypersil Gold aQ 2.1 X 50 mm column (Thermo Scientific, Waltham, MA), equilibrated in 98% buffer A (0.1 % formic acid in water), 2% buffer B (0.1% formic acid, 85% acetonitrile, 10% isopropanol, and 5% water) at a flow rate of 0.4 ml/min (using an Acquity ultra-performance liquid chromatography system, Waters Corporation, Milford, MA). After a 0.5 min wash with the same buffers, peptides were eluted with a linear gradient of 2% to 90% buffer B over 1.5 min with a flow rate of 0.4 ml/min. Peptide masses were detected using a Thermo LTQ Orbitrap Discovery mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive ion mode and set to full-scan Fourier-transform mass spectrometry (FTMS) mode and a resolving power of 30,000 full-width at half maximum (FWHM). The LC/FTMS data were collected over a mass range of *m/z* 100 to 2,000.

In Vitro Digestion with Gastrointestinal Hydrolases.

Triplicate reactions were assembled for each protease. For pepsin digestion, 0.2 ml reactions contained either 16 μg linaclotide or 6 μg insulin, 0.1 M KCl, pH 2, and 200 U/ml pepsin. For trypsin, 0.35 ml reactions contained either 16 μg linaclotide or 6 μg

insulin, 0.1 M Tris-HCl, pH 7.5, and 1 μg trypsin. For aminopeptidase, 0.35 ml reactions contained either 16 μg linaclotide or hSTa, 0.1 M Tris-HCl, 5 mM $MgCl_2$, and 2 units aminopeptidase. For chymotrypsin, 0.35 ml reactions contained either 16 μg linaclotide or guanylin, 0.1 M Tris-HCl, pH 7.5, and 10.5 μg chymotrypsin. All reactions were incubated at 37°C and aliquots removed at time 0, 1 h, and 3h and stopped in either 1M ammonium acetate (pepsin) or ice-cold 0.1% formic acid (trypsin, aminopeptidase, and chymotrypsin). The aliquots were analyzed using LC/HRMS as described above.

Carboxypeptidase A. To digest linaclotide with carboxypeptidase A, reactions (25 μl) in assay buffer (pH 8) containing 65 μM linaclotide, and 1 μl of bovine pancreas carboxypeptidase A (0.7 milliunits) were assembled in triplicate and incubated at 37°C for 15 to 360 minutes (min). The reactions were stopped by dilution with 100 μl of ice-cold 0.1% formic acid and immediate freezing at -80°C. A t_0 control reaction contained no carboxypeptidase A and was diluted in formic acid as described above. Aliquots of the samples were then analyzed by ultra performance liquid chromatography/high-resolution mass spectrometry (UPLC/HRMS) as described above. Control reactions (100 μl) contained N-(4-methoxyphenylazoformyl)-Phe-OH and either no carboxypeptidase A or 2 μl of carboxypeptidase A (1.4 milliunits) were incubated at 37°C for 30 min, diluted with 100 μl of 1 M Na_2CO_3, followed by measurement of the decrease in absorption at 350 nm (A_{350}).

Digestion of linaclotide in the presence and absence of DTT. Linaclotide (65 μM) was incubated in the presence or absence of 5 mM dithiothreitol (DTT) in simulated fasted intestinal fluid (3 mM sodium taurocholate, 0.75 mM lecithin, 8.7 mM NaOH, 2.9 mM NaH_2PO_4, and 10 mM NaCl, pH 6.5; Marques, 2004) containing either chymotrypsin (30

μg/ml) or 1% pancreatin. After 60 min at 37 °C, iodoacetamide was added to a final concentration of 100 mM. This reaction alkylated the free sulfhydryl groups over the course of 30 min at room temperature in the dark. Aliquots of the reaction mixtures were analyzed by liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) as described below.

Liquid chromatography-time of flight mass spectrometry (LC-TOF/MS). Aliquots of reactions were applied to an Atlantis dC_{18} 2.1 X 50 mm column (Waters Corporation, Milford, MA), equilibrated in 98% buffer A (0.1 % formic acid), 2% buffer B (0.1% formic acid, 85% acetonitrile, 15% methanol) at a flow rate of 0.3 ml/min (using an Alliance HT 2795 high-performance liquid chromatography system, Waters Corporation, Milford, MA). After a 4 min wash with the same buffers, peptides were eluted with a linear gradient of 2% to 40% buffer B over 38 min with a constant flow rate of 0.3 ml/min. Peptide masses were detected using a Micromass Q-Tof 2 instrument equipped with an electrospray ionization (ESI) source operating in positive ion mode. LC-TOF/MS data were collected over a mass range of *m/z* 100 to 1000.

LC/MS/MS Method for Linaclotide and MM-419447 in Plasma. Linaclotide and MM-419447 powder were used to create nine mixed-analyte (linaclotide and MM-419447) calibration standards in rat plasma ranging from 2.00 to 500 ng/ml and one linaclotide-only calibration standard at 1.00 ng/ml. A mixed-internal standard (IS) solution containing 100 ng/ml of MM-420026 and MM-437150 was created in phosphate-buffered saline. Each calibration standard and study sample (40-μl aliquots) of the IS solution were mixed with 20 μl of the IS solution. The plasma calibration standards and study samples were processed using solid-phase extraction (SPE) on

Waters MAX 30-µm microelution plates (Waters Corporation Milford, MA). The plates were prepared by preconditioning each well with 200 µl of methanol and two 200-µl aliquots of 10 mM ammonium acetate. The samples were loaded onto the plates in 40-µl aliquots, washed sequentially with 200 µl of 10 mM ammonium acetate, 200 µl of ethyl acetate, and 200 µl of 20% methanol. After washing, the analytes were eluted from the SPE plates using 200 µl of 5/25/70 (v/v/v) formic acid/water/methanol followed by two 200-µl aliquots of 5/15/80 (v/v/v) formic acid/water/methanol. After elution, the samples were evaporated to dryness, reconstituted in 100 µl of 0.2% formic acid in 20% methanol, and analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS). For analysis of samples from the systemic and portal vein concentrations of linaclotide and MM-419447 following oral administration of linaclotide, the method used an Agilent 1200 high performance liquid chromatography (HPLC) system coupled to an AB SCIEX API 4000 triple quadrupole mass spectrometer. For analysis of samples from the oral bioavailability of MM-419447 in rat study, the method used a Waters 1525 binary HPLC and 2777 Sample Manager system coupled to a Waters Quattro Micro triple quadrupole mass spectrometer.

Determination of Linaclotide and MM-419447 Recovered from Feces. For LC/MS/MS determination of linaclotide and MM-419447, fecal pellets were spiked with 300 ng of ^{13}C, ^{15}N-linaclotide internal standard (MM-420026) and allowed to dry at room temperature for 30 min. The pellets were then resuspended in 10 ml/g PBS buffer and homogenized with an Omni homogenizer at maximum speed. After centrifugation, 0.25 ml homogenate was loaded onto an Oasis Max microelution plate (Waters Corporation, Milford, MA). The wells in the plate were washed one time with 0.2 ml of 2% ammonium hydroxide followed by a single wash with 20% methanol in water. Linaclotide and MM-419447 were eluted in 4 steps; two 25 µl elutions with elution buffer A (5/25/70 formic acid/water/methanol, v/v/v) followed by two 50 µl elutions with elution buffer B (5/15/80 formic acid/water/methanol, v/v/v). The samples were evaporated under nitrogen to dryness, reconstituted in 75 µl of 20% methanol in water, and a 40 µl aliquot was analyzed by LC/MS/MS with the method described above.

Metabolism of Linaclotide In Vivo in Surgically Ligated Rat Small Intestinal Loops. The stability of linaclotide in rat intestine was assessed by injecting linaclotide directly into isolated duodenal, jejunal, and ileal loops (1 to 3 cm in length) in male CD rats. The loops were surgically ligated following a modification of the method of London et al. (1997) and injected with 0.2 ml of linaclotide (2.5 µg) in 20 mM Tris-HCl, pH 7.5. Upon completion of the loops, the abdominal wall and skin were sutured. At the indicated times, the rats were sacrificed, the loops were excised, and the fluid from them was recovered at the following time intervals: 2 min (Rat 1), 5 min (Rat 2), 15 min (Rat 3), 45 min (Rat 4) and 90 min (Rat 5) post-injection. The intestinal loop contents were collected

on ice and then stored at -20°C before analysis by radioimmunoassay, as described in Supplemental Methods.

Metabolism of Linaclotide In Vitro in Rat and Mouse Small Intestinal Fluids. To collect intestinal fluid samples for in vitro incubations, surgically ligated female rat duodenal loops or mouse (unknown sex) jejunal loops were prepared as described above and injected with 0.2 ml of Krebs-Ringer solution containing 10 mM glucose and 10 mM HEPES, pH 7.0 (KRGH). The abdominal wall and skin were sutured and the animals were allowed to recover for 30 min. Following recovery, the loops were excised and the fluid was removed and stored at -20°C. Linaclotide (40 µg/ml) was incubated in 25 µl of intestinal fluid at 37°C for varying amounts of time. The reactions were stopped with an equal volume of ice-cold 12% trichloroacetic acid (TCA), vortexed, and centrifuged at 12,000 x *g* for 5 min at 4°C. Quantitative determination of linaclotide and MM-419447 was made using LC/MS/MS as described above.

Metabolism of Linaclotide In Vitro in Human Intestinal Fluid. Samples of human (unknown sex) jejunum luminal contents were obtained from Analytical Biological Services, Inc., (Wilmington, DE). Donor samples were collected 5 h post-mortem. The contents were obtained from a region of the small intestine immediately downstream from the ligament of Treitz. Luminal contents were received frozen in dry ice and stored frozen at -80°C. To prepare the contents to assay linaclotide degradation, the samples were thawed on ice and centrifuged at 16,000 x *g* for 15 to 30 min at 4°C to pellet any solid particles. The resulting supernatant was collected and stored at -80°C. The linaclotide metabolism reactions were carried out at 37ºC in 20 µl of human small intestinal luminal supernatant. The initial concentration of linaclotide was 100 µg/ml

(65,500 nM). At several time intervals (0, 1, 2, 4, and 24 hr), the reactions were diluted with 80 µl of PBS followed by the addition of 100 µl of ice cold 12% TCA to precipitate the proteins. The quenched solution was then vortexed, immediately centrifuged at 16,000 x *g* for 5 min at 4ºC, followed by LC/MS/MS analysis.

Detection of Reduced forms of Linaclotide and MM-419447 after Incubation of Linaclotide in Rat Intestinal Fluid. In order to reduce the rate of linaclotide metabolism in rat intestinal fluid, reactions were carried out in a cocktail containing 10% (v/v) loop intestinal fluid and 90% (v/v) loop fluid without proteins, which was obtained by ultrafiltration of loop fluid through a device that allowed molecules smaller than 1 kDa to pass through (Pall Life Sciences, Exton, PA). This filtrate contained 90% of the thiol measured in loop fluid but very little protein. Incubation reactions (50 µl) contained 65.5 µM of either linaclotide or [$^{13}C_1$]-Ala-linaclotide and were incubated for 15 min at 37°C. After incubation, the reactions were stopped by addition of 50 µl of acetonitrile, followed by addition of iodoacetamide to 50 mM and incubation at room temperature for 30 min in the dark to alkylate the sulfhydryl groups. Aliquots of each reaction were analyzed by LC-TOFMS..

Detection of Small Peptide Fragments of Linaclotide as Degradation Intermediates. Reactions were carried out in a cocktail that contained 20% (v/v) loop fluid and 80% loop fluid filtrate (described above). The 50 µl reactions contained 65.5 µM linaclotide or [$^{13}C_1$]-Ala-linaclotide and were incubated from 15 to 2 hours at 37°C. The reactions were stopped by placing them on ice and removing proteins from the reaction mixture using a 10 kDa ultrafiltration device. The filtrate was adjusted to 20 mM DTT and incubated at 37°C for 30 min to reduce all peptides, followed by alkylation with

100 mM iodoacetamide and incubation at room temperature for 30 min in the dark. For LC-TOFMS analysis, 0.1 ml of 0.1% formic acid was added per 25 µl reaction and 25 µl of this final mixture was injected for analysis as described in Supplemental Methods. Peptides were identified by comparing data from the linaclotide and [$^{13}C_1$]-Ala-linaclotide incubation samples. Ion chromatograph peaks with identical retention times but masses differing by one Da were mapped to predicted masses for fragments with 1-14 amino acids, 0-3 reduced disulfide bonds, and 0-6 alkylated cysteines. Any fragments that could not be identified by mass alone were sequenced by LC/MS/MS.

Complete Digestion of Linaclotide to Single Amino Acids in Rat Intestinal Fluid. Ligated intestinal loop fluid was concentrated to 1/2 or 1/3 the original volume by application to a Nanosep 10K Omega ultrafiltration device (Pall Life Sciences) as indicated by the manufacturer. Each 50-µl digestion reaction contained 0.2 mg/ml peptide (0.131 mM) and 5 µl of concentrated loop fluid, and was supplemented with 2 mM reduced glutathione and 1 mM NADPH. The reactions were incubated at 37°C for 23 hr to achieve complete peptide digestion and were stopped by removal of protein in a Nanosep 10K Omega ultrafilatration device followed by a 1.25-fold dilution into a mixture containing 0.1 mM amino acid standards in order to boost signal. The amino acids were then derivatized and quantitated using the AccQ·Tag system (Waters Corporation, Millford, MA).

Extraction of Linaclotide from Rat Intestinal Fluid. Linaclotide was extracted from ligated loop contents by a modification of the method of Cohen and Gianella (1992). One hundred (100) µl of loop contents were mixed with an equal volume of 12% ice-cold trichloroacetic acid. The mixture was vortexed and immediately centrifuged at 16,000 x *g*

for 5 minutes at 4ºC. The supernatant was then diluted with an equal volume of 10 mM ammonium acetate (pH 5.8) and applied to an SPE plate (SPEC C_{18} Varian A59603, 15 mg sorbent per well), previously prepared as recommended by the manufacturer. After application of the supernatants, the wells were washed with 0.25 ml of 10 mM ammonium acetate (pH 5.8) followed by elution with 0.25 ml of methanol. The samples were dried in a centrifugal evaporator and reconstituted in 0.2 ml of PBS/10% fetal bovine serum buffer. Standard curves for radioimmunoassay analysis were constructed by extracting known amounts of linaclotide as described above. For the quantitation of linaclotide by radioimmunoassay, individual reactions (0.2 ml) containing PBS with 10% fetal bovine serum, linaclotide standards (0 to 300 nM), and extracted samples were mixed with 5 µl of diluted antibody to pSTa (1:40,000 final dilution, 0.0022 µg) and were incubated for 1 to 4 h at 4ºC. One tube contained no linaclotide (the zero standard, B_0) and another no standard and no antibody (non-specific binding, NSB). Labeled tracer pSTa (0.018 µCi, diluted in PBS with 0.1% BSA) was then added and incubated at 4ºC for 12 to 18 h. The antibody-bound fraction containing linaclotide was collected by magnetic separation using 10 µl of sheep anti-mouse IgG beads previously washed twice in 10 volumes of PBS containing 10% fetal bovine serum. The beads were then washed twice with 1 ml of PBS containing 0.1% BSA, collected by magnetic separation, resuspended in 0.1 ml of PBS/0.1% BSA, and added to 2 ml of scintillation fluid. Radioactivity was measured in a LS 6500 scintillation counter (Beckman-Coulter). The radioimmunoassay was able to detect only linaclotide but not MM-419447.

Quantitative Determination of Linaclotide and MM-419447 after Incubation in Rat Intestinal Fluid. Stock solutions (10 µM) of linaclotide and MM-419447 were prepared

in PBS and serially diluted for the standard curves. The quenched reactions were diluted in 0.05% trifluoroacetic acid and 40 µl were analyzed as described above.

Analysis of Intermediates of Linaclotide Metabolism in Rat Intestinal Fluid. Aliquots of reaction mixtures were analyzed by LC-TOF/MS as described above.

Competitive Radioligand Binding Assays Using Rat Intestinal Membranes. Mucosal cells were obtained from the small intestines of two Sprague-Dawley rats by a modification of the method of Kessler et al. (1970). Regions from the small intestine, downstream from the ligament of Treitz, were removed and briefly rinsed with PBS (pH 7.4) containing protease inhibitors (Protease Inhibitor Cocktail, set 1 Calbiochem, EMD Biosciences, La Jolla, CA). The small intestine was cut in segments 6 to 8 cm long and placed in a 100-mm plastic dish containing 10 ml of PBS supplemented with protease inhibitors. A P1000 pipet tip (VWR International, West Chester, PA) was pressed against each segment several times to extrude the mucosa, which were collected by centrifugation (1,000 x *g*), washed twice in 20 ml of PBS, and stored at -20°C. Rat intestinal brush-border membranes were prepared from the mucosa by a modification of the method of Cohen et al. (1986). The frozen mucosa were thawed on ice, resuspended in 40 ml of 2 mM Tris-HCl and 50 mM mannitol (pH 7), and homogenized using an Omni Homogenizer (Model TH, Omni International, Kennesaw, GA) at maximum setting for two min. The resulting homogenate was brought to a concentration of 10 mM calcium chloride ($CaCl_2$). The suspension was mixed for 15 min at 4°C, and then centrifuged at 2,000 x *g* for 10 min. The pellet was discarded and the supernatant was further centrifuged at 19,200 x *g* for 20 min to pellet the brush-border membranes. The membranes were resuspended in 0.5 ml of ice-cold 20 mM HEPES / Tris-HCl buffer (pH

7.2) containing 160 mM NaCl and 5% glycerol, for a final protein concentration of 10 mg/ml. The suspensions were frozen in liquid nitrogen and stored at -80°C. Before use in the binding assay, the frozen membranes were thawed on ice and briefly sonicated by two five-second pulses using a Branson model 450 digital sonifier (Branson Ultrasonic Corporation; Danbury, CT) set at 15% amplitude. Protein determinations were made using the method of Bradford (1976), with gamma globulin as the standard (Bio-Rad Laboratories; Hercules, CA). Duplicate 50-µl reactions were assembled that contained 0.01 to 1,000 nM linaclotide or MM-419447, 0.2 nM [^{125}I]-pSTa (50,000 cpm), and 50 µg of membrane protein in DMEM with 20 mM HEPES (pH 7.0) and 0.2% bovine serum albumin (BSA). Non-specific binding of the radioligand (NSB) was measured in the reactions containing 1,000 nM linaclotide or MM-419447. The maximum specific binding (B_0) of the radioligand was taken to be the binding in the presence of the lowest concentration of competitor tested in this assay (0.01 nM). After incubation at 25°C for 60 min, the binding reactions were applied to Whatman GF/C glass-fiber filters pretreated with 1% polyvinylpyrrolidone and vacuum-filtered to trap the membrane-bound material. The filters were then rinsed with ice-cold PBS buffer and the trapped radioligand was measured in a MicroBeta scintillation counter.

References

1. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal Biochem* **72:**248-254.
2. Cohen MB, Giannella RA (1992) Jejunal toxin inactivation regulates susceptibility of the immature rat to STa. *Gastroenterology* **102:**1988-96.
3. Cohen MB, Moyer MS, Luttrell M, and Giannella RA (1986) The immature rat small intestine exhibits an increased sensitivity and response to Escherichia coli heat-stable enterotoxin. *Pediatr Res* **20:**555-560.
4. Kessler JI, Stein J, Dannacker D and Narcessian P (1970) Biosynthesis of low density lipoprotein by cell-free preparations of rat intestinal mucosa. *J Biol Chem* **245:**5281-5288.
5. Marques M **(**2004) Dissolution Media Simulating Fasted and Fed States. *Dissolution Technologies* **11:**16.

Supplemental Table

TABLE ST1
Complete digestion of linaclotide, MM-419447, and MM-421522 to amino acids

	Concentration of amino acids recovered (μM) % of theoretical complete digestion					
	Linaclotide		MM-419447 (des-Y linaclotide)		MM-421522 (CCNPACTGCY)	
Amino acid	μM	%	μM	%	μM	%
Tyrosine (Y)	251.7	96	40.3	107	127.3	97.1
Alanine (A)	120.8	92.1	137.1	104.6	112.7	86
Proline (P)	127.6	97.3	137.5	104.9	124.1	94.7
Asparagine (N)	118.5	90.4	134.3	102.4	135.4	103.3
Threonine (T)	83.2	63.5	95.6	72.9	98.5	75.1
Glutamic acid (E)	27.3	20.8	43.2	33	0	0
Glycine (G)	48.7	37.1	61.5	47	106.5	81.2
Cysteine (C)	135.6	17.2	150.3	18.9	142.2	27.1

Supplemental Figures

Figure S1

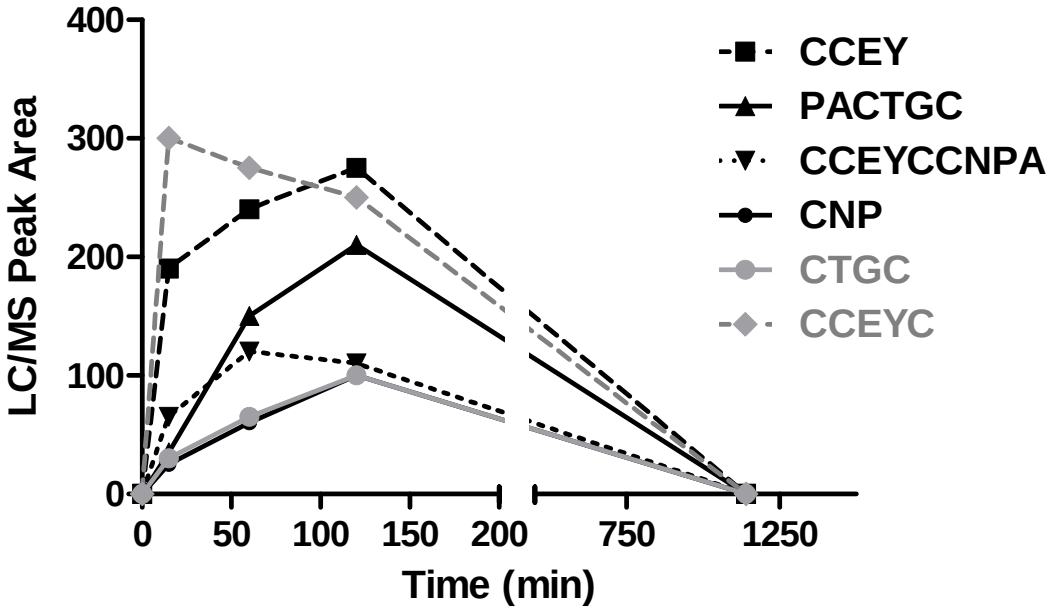

Fig. S1. Appearance and degradation of selected fragments of linaclotide metabolized in rat intestinal fluid. Linaclotide was incubated in diluted intestinal fluid for 15, 60, and 120 min, and overnight. At each time point, proteins were removed and the disulfide bonds in peptides were reduced with DTT followed by alkylation with iodoacetamide. The resulting peptides were identified and their relative concentrations were estimated by LC-TOF/MS.

Figure S2

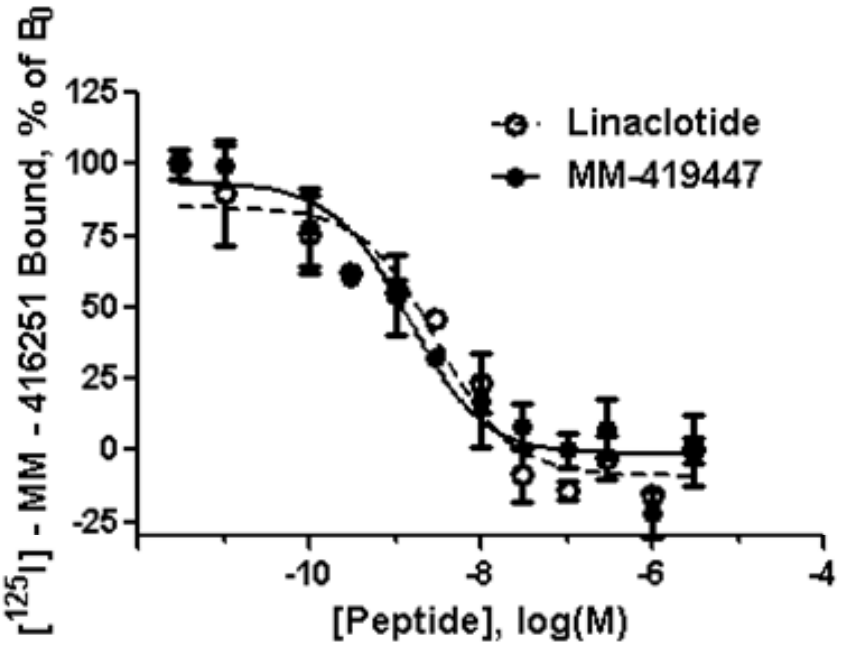

Fig. S2. Competitive radioligand binding assay of linaclotide and MM-419447. Human T84 cells were incubated at pH 7 for 1 hour at 37°C with [^{125}I]-pSTa and increasing concentrations of MM-419447 or linaclotide. Specific binding (%) was obtained by dividing the specifically bound [^{125}I]-pSTa at each MM-419447 and linaclotide concentration by the specifically bound [^{125}I]-pSTa in the absence of either MM-419447 or linaclotide (B_0). All data are expressed as the mean ± S.E.M (n = 2 per binding reaction).

Figure S3

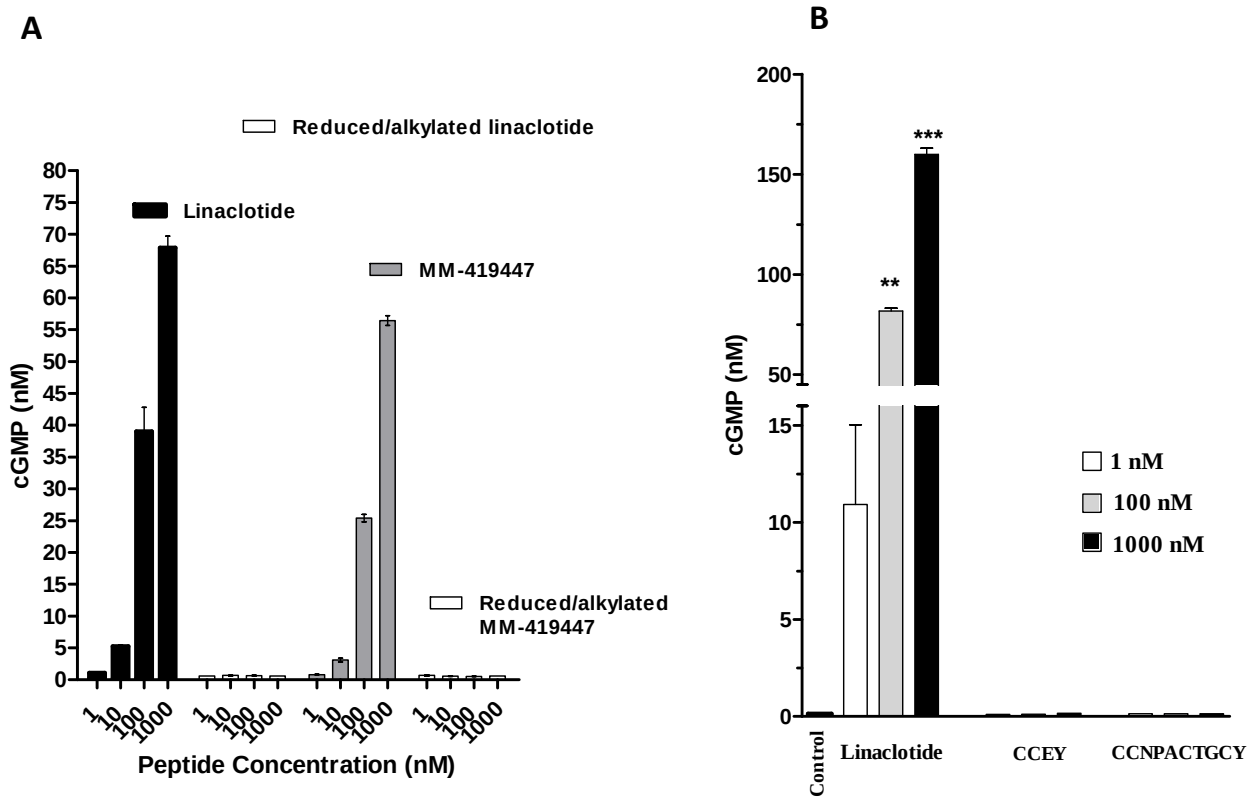

Fig. S3. Pharmacological activity of linaclotide, MM-419447, reduced and alkylated peptides, and linaclotide fragments. T84 cells were incubated with increasing concentrations of the following peptides: (A) linaclotide, reduced and alkylated linaclotide, MM-419447, and reduced and alkylated MM-419447. (B) linaclotide, CCEY, and CCNPACTGCY. Cells were incubated with the indicated peptides for 30 min followed by cell lysis and cGMP determination. Data are expressed as the mean of three (A) and two (B) independent assays. Statistical significance of the cGMP concentrations in cells incubated with linaclotide versus the corresponding cGMP concentrations from

cells with the peptide fragments was determined with Student's unpaired two-tailed *t* tests. All data are expressed as the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$.