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Linaclotide is a potent and selective guanylate cyclase C agonist that elicits pharmacological effects locally in the gastrointestinal tract

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ABSTRACT

Aims: Linaclotide is an orally administered 14-amino acid peptide being developed for the treatment of constipation-predominant irritable bowel syndrome (IBS-C) and chronic constipation. We determined the stability of linaclotide in the intestine, measured the oral bioavailability, and investigated whether the pharmacodynamic effects elicited in rodent models of gastrointestinal function are mechanistically linked to the activation of intestinal guanylate cyclase C (GC-C).
Main methods: Linaclotide binding to intestinal mucosal membranes was assessed in competitive binding assays. Stability and oral bioavailability of linaclotide were measured in small intestinal fluid and serum, respectively, and models of gastrointestinal function were conducted using wild type (wt) and GC-C null mice.
Key findings: Linaclotide inhibited in vitro [^{125}I]-STa binding to intestinal mucosal membranes from wt mice in a concentration-dependent manner. In contrast, [^{125}I]-STa binding to these membranes from GC-C null mice was significantly decreased. After incubation in vitro in jejunal fluid for 30 min, linaclotide was completely degraded. Pharmacokinetic analysis showed very low oral bioavailability (0.10%). In intestinal secretion and transit models, linaclotide exhibited significant pharmacological effects in wt, but not in GC-C null mice: induction of increased fluid secretion into surgically ligated jejunal loops was accompanied by the secretion of elevated levels of cyclic guanosine-3′,5′-monophosphate and accelerated gastrointestinal transit.
Significance: Linaclotide is a potent and selective GC-C agonist that elicits pharmacological effects locally in the gastrointestinal tract. This pharmacological profile suggests that orally administered linaclotide may be capable of improving the abdominal symptoms and bowel habits of patients suffering from IBS-C and chronic constipation.

Introduction

Guanylate cyclase C (GC-C), which was originally identified as the receptor for bacterial STa enterotoxin, is a transmembrane receptor with intrinsic guanylate cyclase activity that is predominantly expressed on the luminal surface of intestinal epithelial cells (Schulz et al. 1990). The endogenous peptide hormones guanylin and uroguanylin are GC-C agonists that regulate the guanylate cyclase activity of this receptor. Guanylin and uroguanylin are expressed along the longitudinal axis of the gastrointestinal tract and released into the intestinal lumen, but their expression pattern is distinctly different in major segments of the intestine and in different cell types within the mucosa due to their altered activity in the variable pH microenvironments found at the mucosal surface of the intestine (Wiegand et al. 1992; Li and Goy 1993; Fan et al. 1996; Hamra et al. 1996, 1997).

Stimulation of GC-C significantly elevates the intracellular concentration of the second messenger cyclic guanosine-3′, 5′-monophosphate (cGMP), which is involved in the regulation of a broad range of physiological processes, including the activation of cGMP-dependent protein kinase II (PKG-II) (Pfeiffer et al. 1996; Schlossmann et al. 2005). PKG-II phosphorylation regulates the activity of the cystic fibrosis transmembrane conductance regulator (CFTR), an ion channel protein colocalized with PKG-II at the apical surface of intestinal epithelial cells (Seidler et al. 1997; Vaandrager et al. 1998). Thus, the activation of GC-C by guanylin and uroguanylin provides an intrinsic mechanism for the control of intestinal fluid homeostasis. This balance is achieved through the stimulation of transepithelial secretion of chloride (Cl^-) and bicarbonate (HCO_3^-) ions and concomitant inhibition of sodium (Na^+) absorption through a blockade of Na^+/H^+ exchange, which results in decreased Na^+ absorption and increased secretion of water into the intestinal lumen (Forte 1999; Vaandrager 2002; Sindic and Schlatter 2006). Studies in GC-C null mice have confirmed the critical role of this receptor in intestinal fluid homeostasis (Mann et al. 1997; Schulz et al. 1997). Thus, the GC-C receptor has the potential as a therapeutic target

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for the treatment of functional gastrointestinal disorders such as constipation-predominant irritable bowel syndrome (IBS-C) and chronic constipation.

IBS-C and chronic constipation are highly prevalent functional gastrointestinal disorders, which occur more frequently in women than men. IBS-C is characterized by symptoms of chronically recurring abdominal pain, discomfort and bloating in association with symptoms of constipation (Drossman 2006; Videlock and Chang 2007). Chronic constipation symptoms include infrequent bowel movements, hard stool, straining during defecation, and a feeling of incomplete evacuation. Abdominal discomfort and bloating may also be present (Sandler and Drossman 1987; Koch et al. 1997). For both disorders, current therapeutic options are limited. Hence, there is a continued medical need for more effective and safer therapeutic agents.

Linaclotide is an orally active 14-amino acid peptide of the guanylin family of cGMP-regulating peptides that also includes the heat-stable microbial ST peptides. In this study, we have investigated whether the pharmacological properties of linaclotide in both in vitro and in vivo models of gastrointestinal function are mechanistically linked to the activation of GC-C.

Materials and methods

Animals

Male and female wild type (wt) and GC-C null (*Gucy2c*$^{-/-}$) mice (C57/BL6) were a gift from Mitchell Cohen (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA). Female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). All animal studies were performed in the laboratories of Ironwood Pharmaceuticals and approved by the Institutional Animal Care and Use Committee.

Reagents

Lyophilized linaclotide was obtained from Polypeptide Laboratories, Torrance, CA, USA. Activated carbon and gum arabic powder were obtained from Sigma, St. Louis, MO, USA. [^{125}I]-STa was prepared by Perkin-Elmer Life and Analytical Sciences (Billerica, MA, USA).

Preparation of intestinal mucosal membranes

Intestinal brush border membranes were prepared from scraped mucosa from four wt and four GC-C null mice based on a method by Sellers et al. (2008). Intestinal mucosal scrapings were homogenized in PBS containing 10% glycerol and protease inhibitors, and stored frozen. Frozen mucosal scrapings were thawed on ice, diluted in 2 mM Tris–HCl, 50 mM mannitol, pH 7.1 with protease inhibitors and sonicated on ice for 0.3-s periods using a 400 W maximum capacity probe sonicator set at 15% amplitude (Branson model 450 Digital Sonifier, 400 W maximum output capacity, Branson Ultrasonics Corp., Danbury, CT). The sonicated material was then centrifuged at 1.600×g for 10 min at 4 °C, and the supernatant collected and centrifuged at 19,000×g for 20 min to recover the membranes. The resulting pellet was resuspended at 12–20 mg/ml protein in ice-cold 20 mM HEPES/Tris buffer, pH 7.2 containing 160 mM NaCl and 5% glycerol.

Intestinal mucosal membrane binding assays

STa(p) was purchased from Bachem America, Inc., radioiodinated (2.200 Ci/mmol, Perkin-Elmer Life and Analytical Sciences, Billerica, MA) 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. The binding reactions were carried out in 50 µl reactions containing 0.1 M sodium acetate pH 5.0, 0.2% BSA, intestinal mucosal membrane protein (50 µg), 53,000 cpm [^{125}I]-STa (11 fmol, 217 pM), and 0.3 nM to 1.0 µM linaclotide competitor. After incubation at 37 °C for 30 min, the reactions were applied to Whatman 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 [^{125}I]-STa radioligand measured in a scintillation counter. Specific binding was determined by subtracting the [^{125}I]-STa bound in the presence of excess unlabeled linaclotide from the total binding. Competitive radioligand-binding curves were generated using GraphPad Prism (GraphPad Software, 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}). Because the [^{125}I]-STa concentration of 217 pM used in these assays was very small compared to its dissociation constant, the calculated IC_{50} and K_i values are in effect identical. The results are expressed as mean ± standard error of the mean (SEM).

Linaclotide metabolism in intestinal fluid

To collect small intestinal fluid samples, two mice were fasted overnight but had access ad libitum to filtered tap water. Laparotomy was performed on anesthetized (isofluorane) mice and their small intestines were exteriorized. The areas of the jejunum selected for ligation were rinsed out with 3 ml of 20 mM Tris–HCl buffer, pH 7.5 prior to the creation of loops 1 to 3 cm in length, which were then injected with 0.2 ml vehicle (Krebs–Ringer solution containing 10 mM glucose, 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 mice were sacrificed, the loops excised, and the fluid removed and stored at −20 °C. Linaclotide (0.1 mg/ml) was incubated in either 0.010 ml of intestinal fluid or 0.010 ml phosphate-buffered saline (PBS) (control) at 37 °C for varying amounts of time. The reactions were stopped by addition of 0.015 ml PBS and one volume of ice-cold 12% trichloroacetic acid (TCA), vortexed and centrifuged at 16,000×g for 5 min at 4 °C. Linaclotide degradation was analyzed by LC–MS/MS using the MassLynx version 4.0 SP4 software for molecular weight prediction and data analysis.

Oral bioavailability of linaclotide in mice

To determine oral bioavailability, three groups ($n=3$) of female CD-1 mice received linaclotide (8 mg/kg) intravenously (i.v.), while two groups ($n=3$) received linaclotide (8 mg/kg) by gavage (p.o.). Blood was allowed to clot for 5 min, centrifuged at 13,000×g for 3 min, and the serum was collected and stored at −80 °C until sample preparation and analysis by LC–MS/MS. The concentration of linaclotide was determined based on a standard concentration curve of linaclotide generated using a set of standards prepared in mouse serum (lower limit of quantitation was 1.0 ng/ml). Data were collected using Waters MassLynx version 4.0 software. Linaclotide serum concentrations were plotted as a function of time using GraphPad Prism 5.0 software. Pharmacokinetic parameters for oral and intravenous administration were calculated using WinNonlin version 5.2. If no analyte was detected, the concentration was set to zero for calculations of the AUC and oral bioavailability.

Intestinal fluid secretion assay

Intestinal loops in wt and GC-C null mice ($n=5$–7/group) were surgically ligated after the mice had been placed under isofluorane anesthesia and laparotomy was performed to exteriorize the small intestine. The small intestines were flushed with Krebs–Ringer buffer (K–R, 10 mM glucose, 10 mM HEPES) pH 7.0 (KRGH) and a loop of approximately 3 cm in length was created halfway between the stomach and the cecum. Loops were injected with either 100 µl linaclotide (5 µg) or 100 µl vehicle (KRGH), and the animals were allowed to recover for 90 min prior to euthanasia. The loops were then