Enhanced Gastrointestinal Motility with Orally Active Ghrelin Receptor Agonists

Soratree Charoenthongtrakul, Derek Giuliana, Kenneth A. Longo, Elizabeth K. Govek, Anna Nolan, Samantha Gagne, Kristen Morgan, Jeffrey Hixon, Neil Flynn, Brian J. Murphy, Andres S. Hernández, Jun Li, Joseph A. Tino, David A. Gordon, Peter S. DiStefano, and Brad J. Geddes


Received December 22, 2008; accepted February 26, 2009

ABSTRACT

The orexigenic peptide ghrelin has been shown to have prokinetic activity in the gastrointestinal (GI) system of several species, including humans. In this series of experiments, we have evaluated the prokinetic activity of novel, small-molecule ghrelin receptor (GhrR) agonists after parenteral and peroral dosing in mice and rats. Gastric emptying, small intestinal transport, and fecal output were determined after intraperitoneal and intracerebroventricular dosing of GhrR agonists, using ghrelin as a positive control. These same parameters were evaluated after oral gavage dosing of the synthetic agonists. Regardless of dose route, GhrR agonist treatment increased gastric emptying, small intestinal transit, and fecal output. However, fecal output was only increased by GhrR agonist treatment if mice were able to feed during the stimulatory period. Thus, GhrR agonists can stimulate upper GI motility, and the orexigenic action of the compounds can indirectly contribute to prokinetic activity along the entire GI tract. The orexigenic and prokinetic effects of either ghrelin or small-molecule GhrR agonists were selective for the GhrR because they were absent when evaluated in GhrR knockout mice. We next evaluated the efficacy of the synthetic GhrR agonists dosed in a model of opiate-induced bowel dysfunction induced by a single injection of morphine. Oral dosing of a GhrR agonist normalized GI motility in opiate-induced dysmotility. These data demonstrate the potential utility of GhrR agonists for treating gastrointestinal hypomotility disorders.

Neuroendocrine systems function to coordinate complex physiological and homeostatic processes, and the peptide hormone ghrelin is a key factor in the neuroendocrine control of nutrient metabolism. Ghrelin is secreted primarily by the stomach and reaches peak plasma levels in anticipation of a meal. It then acts at its specific G-protein-coupled receptor (GhrR, also referred to as GHSR-1a) to potently stimulate food intake (FI) and nutrient storage (Guan et al., 1997; Leite-Moreira and Soares, 2007). Thus, ghrelin is both anatomically and physiologically positioned to play a significant role in the regulation of metabolism (Inui et al., 2004). Consistent with its role in determining the fate of consumed nutrients, ghrelin has a potent prokinetic action on the gastrointestinal (GI) tract. The orexigenic and prokinetic action of ghrelin has been established firmly in rodents (Wren et al., 2000; Dornonville de la Cour et al., 2004; Fukuda et al., 2004; Druce et al., 2005; Kitazawa et al., 2005) and humans (Wren et al., 2001; Murray et al., 2005; Tack et al., 2005; Binn et al., 2006). Ghrelin stimulates the migrating motor complex, resulting in increased gastric emptying and upper GI tract motility in a variety of species (Fujino et al., 2003; Tack et al., 2006; Wang et al., 2007). Despite 50% structural homology between ghrelin and the prokinetic peptide hormone motilin, the activity of these two hormones has been shown convincingly to function via distinct receptors (Depoortere et al., 2003; Sanger, 2008). The ability to stimulate hunger, FI, and GI motility suggests that ghrelin agonists may represent an attractive and unique therapeutic strategy for the treatment of motility disorders (Sanger and Alpers, 2008), such as diabetic and idiopathic gastroparesis, postoperative ileus, opiate-induced bowel dysfunction, and chronic constipation of idiopathic origin (Murray et al., 2005; Levin et al., 2006; Qiu et al., 2008b,c).

Selective, small-molecule GhrR agonists currently are be-
ing evaluated clinically as potential therapeutics for GI dysmotility disorders (Venkova et al., 2007). Parenteral dosing with synthetic, macrocyclic GhrR agonists successfully stimulated GI motility in rodent models (Venkova et al., 2007). However, although there is clinical potential for parenteral dosing in certain indications such as postoperative ileus, the oral dose route offers broader therapeutic utility. We have developed potent, orally bioavailable, dimethyl glycyl-O-benzyl-serine GhrR agonists (Li et al., 2005) and have evaluated them for their ability to affect motility throughout the GI axis in rodents after both parenteral and peroral dosing. We report here that the small-molecule ghrelin agonists, Elixir (EX)-1314 and EX-1315, stimulate gastric emptying, small intestinal transit, and fecal output in addition to stimulating food intake. In addition, these compounds ameliorate opiate-induced bowel dysfunction, supporting the potential use of these agents in a range of GI dysmotility disorders.

Materials and Methods

General. The outline and rationale for testing the efficacy of our agonists on GI function is as follows. Initial experiments were performed to determine the ability of two representative members of the dimethyl glycyl-O-benzyl-serine series of ghrelin agonists to increase the most proximal components of GI physiology, gastric emptying, and small intestinal transit (SIT). In all cases, preliminary studies were conducted to determine optimal doses and dose timing. Intraperitoneal dosing of compounds was employed for initial studies so that the effects could be compared with ghrelin, which can only be administered parenterally. We then evaluated whether we could recapitulate these actions when the agonists were dosed by oral gavage. Furthermore, we tested whether the prokinetic action of GhrR agonists on the upper GI could lead to increased motility along the entire GI tract by measuring fecal output. Here, ghrelin was not used as a positive control because it is not orally absorbed. Next, the receptor selectivity of the compounds for SIT and FI were determined in mice lacking the GhrR. These experiments also were carried out by intraperitoneal dosing so that ghrelin could be used as a comparator. We then examined the ability of compounds to act via central sites of action by administering the compounds intracerebroventricularly and measuring food intake and GI motility versus ghrelin. Finally, we tested the efficacy of compounds in mice in a model of opiate-induced GI dysmotility to determine the effectiveness of the small-molecule GhrR agonists under conditions of GI pathology.

Compounds. Acylated ghrelin (octanoyl-Ser3-ghrelin) is the active form of the peptide and was purchased from Phoenix Pharmaceuticals (Belmont, CA). Human motilin was purchased from Sigma-Aldrich (St. Louis, MO). In vitro pharmacologic and pharmacokinetic properties of small-molecule GhrR agonists (Li et al., 2005) were assessed by standard methods (see Supplemental Table 1). Morphine sulfate was purchased from Hospira, Inc. (Lake Forest, IL).

Animals. Use of experimental animals for all protocols was approved by the Elixir Pharmaceuticals Institutional Animal Care and Use Committee (Cambridge, MA) in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Lean, male C57BL/6 mice were purchased from Taconic Farms (Germantown, NY), and intracerebroventricularly cannulated male, C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A colony of GhrR knockout (KO) and wild-type (WT) mice (Sun et al., 2004) was maintained at Charles River Laboratories, Inc. (Wilmington, MA) and transferred to Elixir for experiments. Mice from the GhrR KO colony were genotyped using a polymerase chain reaction assay followed by gel electrophoresis. Before use in experiments, all mice were acclimated to facility conditions for 5 days, housed in a controlled environment (22°C, −40% humidity, 12:12-h in-phase light/dark cycles at 7:00 AM/7:00 PM) on ventilated racks (Thoren Caging Systems, Inc., Hazleton, PA). Rat studies were conducted using male Sprague-Dawley (SD) rats (250–300 g; Rhoeoseira A/S, Roedovre, Denmark). Unless otherwise noted, animals were individually housed and maintained on a normal chow diet (PicoLab rodent diet 20; Purina, St. Louis, MO) with ad libitum access to food and water. In experiments involving the transfer of animals to a novel, clean cage, a small quantity of home cage bedding was also transferred to the new cage to minimize stress. Animals were approximately 12 weeks of age at the start of experiments.

Gastric Emptying. Gastric emptying after treatment with GhrR agonists was determined in both mice and rats. In mice, gastric elimination was measured by weighing stomach contents after oral gavage of a charcoal marking mixture (Mascolo et al., 2002; Navarro et al., 2006). In rat studies, gastric elimination of an acaloric mixture containing phenol red (Qiu et al., 2008b) was measured spectrophotometrically.

Mice were weighed and sorted into equivalent groups according to body weight (BW) and then fasted overnight with free access to water. The gastrointestinal transit assay was then conducted according to standard methods, described previously (Mascolo et al., 2002; Navarro et al., 2006). On the morning of the experiment, water was also removed from all cages. The duration of fasting and water restriction was 18 and 4 h, respectively. Mice were divided into groups (n = 6/group for the EX-1315 experiment, n = 16/group for the EX-1314 experiment) of equal body weight, each receiving an intraperitoneal injection of vehicle (phosphate-buffered saline) or 500 μg/kg ghrelin, EX-1314, or EX-1315 in a dose volume of 5 ml/kg. Five minutes later, each mouse was given an oral dose (250 μl) of a black, marking mixture consisting of 10% activated charcoal and 5% gum arabic in distilled H2O (Sigma-Aldrich). Fifteen minutes later, mice were sacrificed, whereupon the stomachs were excised immediately distal to the cardiac and pyloric sphincters, and the contents were extracted and weighed. A separate, baseline group, was given the oral charcoal mixture and sacrificed immediately, and stomach content weights were measured. Percentage gastric emptying (GE) was calculated as follows:

\[
\%GE = \frac{\text{mean } t = 15 \text{ min stomach content weight of experimental group} - \text{mean stomach content weight of baseline group}}{\text{mean stomach content weight of baseline group}} \times 100
\]

GE assays were also conducted in SD rats (n = 6/group) after intraperitoneal and oral gavage dosing with EX-1314. These studies were conducted as described above, except that the GI marking mixture consisted of 1.5 ml of a 1.5% hydroxypropyl-methyl-cellulose solution (noncaloric) containing 0.05% phenol red as an indicator. In addition, in the oral gavage study, vehicle or EX-1314 (300 mg/kg; 2.5 ml/kg) were dosed 45 min before GI marking mixture dosing. Rats were sacrificed at 5, 10, and 20 min after the oral marking mixture load. The stomach was removed as described above, and the contents were emptied into 50 ml of a 0.1 M NaOH solution. Particles were removed by centrifugation (2000g, 2 min), and absorbance of the supernatant at 560 nm was read on a spectrophotometer (Qiu et al., 2008a). The fraction remaining was determined as follows:

\[
\%GE = \frac{\text{mean absorbance of experimental group at a given time point} - \text{mean absorbance obtained from baseline group}}{\text{mean absorbance of experimental group}} \times 100
\]

SIT. Mice were fasted overnight for 18 h. After the fast, all mice were weighed and sorted into equivalent groups (n = 6/group) according to BW. Mice were then dosed with vehicle, ghrelin, or a GhrR agonist. Preliminary experiments determined the optimal time courses for the different routes of administration. Depending on the route of compound administration, the mice were then dosed with a
predetermined amount of the black marking charcoal mixture (as per mouse GE assay), 1 (intracerebroventricular study), 15 (intraperitoneal study), or 45 (per os study) min later. Fifteen minutes after the charcoal bolus, the mice were euthanized via CO2 asphyxiation. The stomach and small intestines were excised, and the total length of the small intestine, beginning with the pyloric sphincter to the distal end of the ileum, was recorded. Percentage small intestinal transit (%SIT) was calculated using the following formula: %SIT = (distance the charcoal smear traveled)/total length of the small intestines) x 100.

Feeding Effect on Fecal Output. We evaluated whether the prokinetic actions of our GhrR agonists cold lead to fecal output and whether GhrR-induced FI would influence this action. Mice were fed ad libitum until the day of the assay. Then, to eliminate variability in stomach contents between individual mice, food and water were removed from their cages 2 h before the experiment, starting at 10:00 AM. Because mice feed and drink very little during the daylight hours, it was believed that this would make a minimal impact on their general physiology or colonic fecal content. Mice were dosed (n = 6/group) with either vehicle or EX-1314 (300 mg/kg; 2.5 ml/kg) by oral gavage and then placed in a clean cage (i.e., free of feces). The food hopper of each cage contained a known, excess amount of food. Because ghrelin is not orally bioavailable, it was not used as a positive control in these studies. Food and water could then be consumed ad libitum, and the quantity of food remaining could be measured at a given time after treatment. Immediately after dosing, half of the mice remained without food or water for 4 h, while the other half was able to feed ad libitum for 4 h. Fecal pellet number, total fecal mass, and cumulative FI were measured 4 h after dosing.

GhrR Knockout Mouse Studies. To determine the selectivity of the actions of synthetic GhrR agonists, groups of GhrR KO and WT littermates were evaluated for their response to agonist treatment in both feeding and SIT assays. For both studies, mice were dosed intraperitoneally with vehicle or 500 μg/kg of either ghrelin or EX-1314. For the feeding assays, nonfasted mice (n = 8/group) were sorted into equivalent groups according to their morning BW. After the sort, all mice were dosed intraperitoneally and returned to their cage. The GhrR dependence of the prokinetic action of GhrR agonists was also determined by conducting an SIT assay on groups of GhrR KO and WT littermates (n = 6/group) as described above.

Direct CNS Dosing Studies. Intracerebroventriculally cannulated mice were acclimated to handling for 3 days before an experiment. On the morning of each study, ghrelin and EX-1314 were formulated in artificial cerebrospinal fluid (pH 6.4; Harvard Apparatus Inc., Holliston, MA). Initial dose-response experiments were carried out for ghrelin and EX-1314 to determine optimal doses (data not shown). Vehicle (artificial cerebrospinal fluid), 0.25 μg/ml ghrelin, or 0.0025 μg/ml EX-1314 was infused in conscious animals (n = 7/group) at a rate of 1 μl/min for 4 min so that a total of 1 μg of ghrelin or 0.01 μg of EX-1314 was administered to the animals. The intracerebroventricular dosing study was conducted in two phases. The first phase of the study evaluated the feeding and fecal output responses of animals fed ad libitum to an intracerebroventricular infusion of GhrR agonists. On the morning of the study, mice were sorted into equivalent groups according to morning BW. Mice were then dosed and placed in a clean cage (i.e., free of feces), where they continued to have access to food and water ad libitum. Cumulative food intake and fecal output (total fecal pellet number and total fecal mass) were determined 4 and 24 h after compound dosing. Body weight was again measured 24 h after dosing. Phase 2 of the intracerebroventricular dosing study was performed after a 4-day washout period, with mice remaining in the same treatment groups as in phase 1. Mice were fasted overnight, dosed intracerebroventricularly, and an SIT assay was performed (as described above).

Comparative Pharmacology Studies. The next experiments were designed to evaluate the relative sensitivity of mice to the actions of synthetic GhrR agonists. Groups of nonfasted, male mice (n = 8/group) were dosed orally by gavage (5 ml/kg) with either vehicle (phosphate-buffered saline) or EX-1314 (1–1000 mg/kg) and then placed in a clean cage (i.e., free of feces), where they had access to food and water ad libitum. One hour after dosing, FI and fecal output were measured as described above. A third cohort of mice was given the same doses (n = 4/group) and then sacrificed by CO2 asphyxiation 1 h later for compound exposure determination. Blood was collected from this third cohort via cardiac puncture for determination of plasma concentrations of EX-1314 by a liquid chromatography/mass spectrometry method (Absorption Systems, Exton, PA).

Opiate-Induced GI Dysmotility. All studies conducted thus far were performed using rodents in their normative state. Therefore, we next determined whether our GhrR agonists could affect GI motility in a model of opiate-induced bowel disorder (Greenwood-Van Meerveld et al., 2004; Venkova et al., 2007). On the day before the experiment, lean male C57BL/6 mice (n = 8/group) were fasted overnight (18 h), with water available ad libitum. The following morning, mice received a subcutaneous dose of morphine sulfate (3 mg/kg in water) and were transferred to a clean cage. One minute later, animals were dosed by oral gavage with vehicle or EX-1314 (100 or 300 mg/kg). Forty-five minutes after compound treatment, all animals were dosed orally with 500 μl of the charcoal meal. Fifteen minutes later, mice were sacrificed, and the %SIT was determined as described above.

Statistical Analyses. All data are represented as the mean ± S.E. Compound versus vehicle effects were analyzed using a one-way analysis of variance and Tukey’s post hoc test. For experiments comparing the effect of treatment with respect to genotype, a two-way analysis of variance with Bonferroni post hoc tests was used. All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego, CA). Statistical significance was defined as p < 0.05.

Results

Intrinsic Activity of GhrR Agonists. A series of in vitro experiments was carried out to characterize the intrinsic pharmacological properties of small-molecule GhrR agonists (Supplemental Table 1). A prototype of the class of molecules, EX-1315, bound with high affinity to the human GhrR (Kᵢ = 55 nM). EX-1315 also showed potent functional activity, with an EC₅₀ value of 1.5 nM. Likewise, EX-1314 demonstrated high-affinity binding (Kᵢ = 2.3 nM) and potent functional activity (EC₅₀ = 0.4 nM).

Gastric Emptying. Initial experiments sought to establish the extent to which our small-molecule GhrR agonists stimulate gastric emptying in comparison with ghrelin. Intraperitoneal injection of 500 μg/kg of ghrelin, EX-1315 (Fig. 1A), or EX-1314 (Fig. 1B) 5 min before an oral charcoal load resulted in a significant increase in gastric emptying compared with vehicle-treated mice. Likewise, rats dosed with EX-1314, either intraperitoneally (Fig. 1C) or by oral gavage (Fig. 1D), had a significant increase in GE relative to the vehicle group. After intraperitoneal dosing, GE was significantly increased in rats given EX-1314 at 5-, 10-, and 20-min time points. However, in the rats receiving EX-1314 by oral gavage, a significant difference was only observed at the 5-min time point, although the trend of greater GE in the compound group continued at later time points.

Small Intestinal Transit. We evaluated the extent to which GhrR agonism stimulated SIT. After an oral load of a non-nutritive charcoal mixture, mice dosed at 500 μg/kg i.p. with ghrelin, EX-1315, or EX-1314 showed robust and significant stimulation of SIT relative to vehicle-treated controls that were comparable in magnitude (Fig. 2A). To determine
whether the prokinetic action of small-molecule GhrR agonists could be achieved after oral dosing. EX-1315 and EX-1314 were dosed orally at 10, 100, and 300 mg/kg in separate SIT experiments. In vehicle-treated mice, the charcoal admixture traveled approximately 34% of the length of the small intestine. Oral dosing with both EX-1314 (Fig. 2B) and EX-1315 (Fig. 2C) caused an increase in %SIT that was significantly greater than vehicle-treated controls at the 100 and 300 mg/kg doses.

**Feeding Effect on Fecal Output.** We next sought to determine whether the prokinetic action of GhrR agonists was capable of propagating a prokinetic signal along the entire length of the GI tract. To do so, we tested whether dosing of GhrR agonists by oral gavage was also capable of increasing fecal output. In addition, we examined the contribution of feeding to GI motility by comparing fecal output of mice with and without access to food during the prokinetic period. Figure 3 shows the 4-h fecal output data from mice that were dosed orally with vehicle or EX-1314. After a 300 mg/kg dose of EX-1314, mice that were given access to food consumed significantly more food (1.06 ± 0.17 g) relative to vehicle-treated controls (0.35 ± 0.16 g). Furthermore, fecal output in GhrR-treated mice was 2-fold greater versus controls (p < 0.05). It is interesting that mice that were denied access to food showed no increase in fecal output relative to controls, suggesting that GhrR agonist-stimulated fecal output is a consequence of stimulated food intake. The fecal pellets of all groups studied were qualitatively similar, with no evidence of diarrhea.

**Effect of GhrR Agonists in GhrR KO and WT Mice.** To determine the GhrR selectivity of the GhrR agonists, FI was measured (as described above) 4 h after an intraperitoneal injection of vehicle, ghrelin, or EX-1314 in male GhrR KO and WT mice fed ad libitum (Fig. 4). In WT mice, administration of ghrelin or EX-1314 (both at 500 μg/kg) increased FI approximately 2-fold compared with vehicle-treated controls (p < 0.05, Fig. 4A). However, the same treatments administered to GhrR KO mice yielded no significant differences between vehicle-treated and either ghrelin- or EX-1314-treated groups. An SIT assay was also performed on GhrR KO and their WT littermates (Fig. 4B). There was no significant difference in %SIT between GhrR KO and WT littermates treated with vehicle. As observed in previous experiments, both ghrelin and EX-1314 dosed at 500 μg/kg stimulated a significant increase in SIT in WT mice relative to vehicle-treated controls. However, in mice lacking the GhrR, neither ghrelin nor EX-1314 was prokinetic, suggesting that the agonists act exclusively through the GhrR.

**Direct CNS Dosing Studies.** The stimulatory effects of ghrelin on FI and GI motility are thought to be mediated predominantly at the level of the CNS, although actions on peripheral tissues may also be involved (Sakata et al., 2003; Xu et al., 2005; Zhang et al., 2005). To assess the contribution of central action in the effects of our agonists, we infused ghrelin or EX-1314 into the lateral cerebral ventricle of mice fed ad libitum. Four hours after the intracerebroventricular infusion, vehicle-treated mice consumed 0.52 ± 0.19 g of food, but both the ghrelin- and EX-1314-treated mice ate almost three times as much (p < 0.05, Fig. 5A). Figure 5B shows that 24 h after the infusions, FI remained significantly elevated in both the ghrelin- and EX-1314-treated groups, relative to controls.

We also examined the effects of intracerebroventricular ghrelin or EX-1314 administration on fecal output in these animals. Fecal expulsion, both in terms of the number of fecal pellets (Fig. 5, C and D) and the total mass of expelled feces (Fig. 5, E and F), was evaluated 4 and 24 h after intracerebroventricular dosing. As was observed with FI, both ghrelin and EX-1314 treatment significantly increased fecal output (pellet number) by approximately 2.5-fold relative to vehicle...
controls (p < 0.05, Fig. 5C), an effect that was sustained for at least 24 h after intracerebroventricular injection (Fig. 5D).

Similar results were obtained when the fecal output was assessed according to mass (Fig. 5, E and F). The fecal pellets of all groups were qualitatively similar, with no evidence of diarrhea. Despite the increased fecal output that occurred in ghrelin- and EX-1314-treated mice, the short-term consequence of GhrR agonist treatment was a net increase in BW (Fig. 5G). Although vehicle-treated mice gained 0.22 g, mice dosed intracerebroventricularly with ghrelin or EX-1314 gained three to four times as much weight (Fig. 5G) within just 24 h.

As a terminal assessment of the prokinetic action of GhrR
agonists, intracerebroventricularly cannulated mice were redosed according to their original treatment group, whereupon the effect on SIT was evaluated. Mice infused with EX-1314 experienced significant increases in GI motility relative to vehicle-treated controls that were similar to those observed with intraperitoneal injection of the agonists (Fig. 5H). The single dose of ghrelin included in the study failed to increase %SIT to a significant degree.

**Comparative Pharmacology Studies.** We next assessed the relative effectiveness of orally administered ghrelin agonists to stimulate GI motility compared with FI. This study was run in nonfasted mice. Figure 6, B and C, shows the relative potencies of GhrR agonist EX-1314 in stimulating FI and GI motility, respectively. Measures of efficacy and plasma exposure levels were measured at the 1-h time point for direct comparisons. EX-1314 showed a dose-linear increase in plasma concentrations at the 1-h time point (Fig. 6A) and elicited a dose-responsive increase in FI relative to vehicle-treated controls, with a minimum effective dose of approximately 10 mg/kg (Fig. 6B). However, fecal output (Fig. 6C) measured in the same animals did not show a dose response over the 1 to 1000 mg/kg dose range, in that apparent maximal effects were seen with 1 mg/kg.

**Opiate-Induced GI Dysmotility.** To this point, the effects of EX-1315 and EX-1314 had been assessed exclusively in normative animals. Therefore, we determined whether our ghrelin agonists could affect GI motility in a model of opiate-induced bowel disorder in which morphine is used to slow
gastrointestinal transit (Greenwood-Van Meerveld et al., 2004). As shown in Fig. 7, treatment of mice with morphine (morphine/vehicle group) significantly decreased SIT by 40% compared with the vehicle/vehicle control. Oral treatment with EX-1314 was able to ameliorate morphine-induced hypomotility in an apparent dose-responsive manner. When dosed at 300 mg/kg, EX-1314 significantly increased SIT to levels observed for controls, indicating complete reversal of the morphine-induced hypomotility.

**Discussion**

We have demonstrated the utility of a novel class of small-molecule GhrR agonists as prokinetic gastrointestinal agents that are as potent and efficacious as acylated ghrelin. Synthetic GhrR agonists have been shown previously to stimulate GI motility effectively when injected intravenously (Greenwood-Van Meerveld et al., 2004; Heightman et al., 2007; Venkova et al., 2007). Here, we have demonstrated potent efficacy of GhrR agonists on stimulated gastric emptying, small intestinal transit, and fecal output in addition to stimulating food intake. These effects could be produced after intraperitoneal, intracerebroventricular, and peroral dosing. Furthermore, EX-1314 was shown to ameliorate opiate-induced bowel dysfunction in mice after oral dosing, supporting the potential use of these agents in a range of human GI dysmotility disorders.

Ghrelin has been shown to enhance gastric emptying in mice (Dornonville de la Cour et al., 2004; Kitazawa et al., 2005; Qiu et al., 2008a), rats (Fukuda et al., 2004; Ariga et al., 2007), and humans (Tack et al., 2006). Our initial experiments were performed to determine the ability of the dimethyl glycyl-O-benzyl-serine ghrelin agonists to increase the most proximal components of GI physiology, GE, and SIT. In intraperitoneal dosing studies in mice, we found that synthetic GhrR agonists were capable of stimulating GE to the same extent as ghrelin itself (Fig. 1). Similar results were obtained in rats. Consistent with the available literature (Trudel et al., 2002; Kitazawa et al., 2005), GhrR agonist-induced increases in GE seem to be greater in rats relative to mice, although this was not studied exhaustively in the current studies. In addition, we show that GE is enhanced in rats after oral gavage dosing with a synthetic GhrR agonist. The GhrR agonist-induced upper GI motility signal could also be detected by examining SIT after both intraperitoneal and oral gavage dosing (Fig. 2). We found the SIT to have a relatively high degree of interassay variability and a low dynamic range compared with the other assays used in this series of experiments. This may be a consequence of the fact that mice are fasted both before and throughout the assay. Fasting and the inability to feed may decrease overall responsiveness to the prokinetic actions of GhrR agonists (see below). Nevertheless, these assays confirmed that both endogenous and synthetic GhrR agonists increased GI motility after intraperitoneal dosing and that the synthetic agonists were also effective in this assay after oral dosing.

Ghrelin and synthetic GhrR agonists are potently orexigenic (Wren et al., 2000; Figs. 4–6). The small-molecule GhrR agonists examined in this series of experiments are also able to stimulate feeding after oral dosing. We also found that fecal output is robustly stimulated by the synthetic GhrR agonist treatment after oral dosing of compound (Fig. 3). It is important to note that these experiments were not designed to demonstrate a direct effect of GhrR agonists on...
the colon but rather to elucidate whether activation at the level of the upper GI would propagate along the length of the GI tract. We show that GhrR agonists can increase fecal output, but only if mice were able to feed during the stimulatory period. Thus, GhrR agonists can stimulate upper GI motility, and the orexigenic action of the compounds may indirectly contribute to prokinetic activity along the entire GI tract via an indirect mechanism. It is interesting that the increased sensitivity of mice in terms of fecal output and the lack of an apparent dose response (Fig. 6) suggest that relatively small amounts of food intake are required to enhance the GhrR-stimulated fecal output. Moreover, it may be possible to separate the orexigenic and prokinetic actions of GhrR agonist after oral dosing. A comparison of the data in Fig. 6, B and C, suggest that there may be a differential sensitivity to the effects of GhrR agonists on feeding and GI motility. Consistent with food dependence for robust GI motility after GhrR agonist treatment, we note the high degree of interassay variability in the SIT assay, which is run in fasted animals (compare Figs. 2A and 4B). Nevertheless, it is clear that ghrelin agonism is still prokinetic even in the absence of feeding because SIT is enhanced. However, it is also clear that the additional prokinetic stimulation provided by feeding is required for the full expression of GhrR agonist-induced GI motility and for transmission of that signal along the entire length of the GI tract. Therefore, ghrelin may participate in reflex postprandial fecal elimination or the gastrorectal reflex (Masuda et al., 2000). Thus, the data suggest the potential utility of GhrR agonists for the therapeutic relief of both upper and, in nonfasting conditions, lower GI dysmotility.

It has been demonstrated previously that there are no differences in gastric emptying in mice lacking endogenous ghrelin relative to WT littermates (De Smet et al., 2006). Consistent with these observations, we found no significant differences in the GI motility of GhrR KO mice relative to WT littermates, comparing vehicle-treated groups. Likewise, the GhrR agonists tested were without effect in mice lacking the receptor. These findings are consistent with the high degree of in vitro GhrR selectivity observed in counter-screen assays (data not shown), which show a lack of off-target activity of these synthetic molecules. Therefore, we can be confident that stimulation of FI and GI motility after administration of ghrelin and the synthetic agonists described here are specific to actions at the GhrR receptor.

GI motility in response to GhrR agonism is thought to be mediated by both a central action and a direct effect at the level of the enteric nervous innervation of the gut (Fukuda et al., 2004; Inui et al., 2004). Central and peripheral injections of GhrR agonists in rodents have been shown previously to increase GI motility (Tebbe et al., 2005; Shimizu et al., 2006; Fraser et al., 2008). Consistent with this growing body of literature, we show that intracerebroventricular delivery of a small-molecule GhrR agonist potently stimulates FI, SIT, and fecal output. As observed in oral dosing studies, the fact that animals were free to feed ad libitum after intracerebroventricular injections probably allowed the prokinetic signal to manifest as fecal output in these experiments. The results presented here confirm that GhrRs in the CNS contribute to the prokinetic action of GhrR agonists because both ghrelin and EX-1314 robustly stimulated GI motility after intracerebroventricular doses that were several orders of magnitude lower than those needed for peripheral dosing. The single dose of ghrelin dosed in the intracerebroventricular studies failed to increase %SIT to a significant degree. It is tempting to speculate that this may indicate a degree of dissociation of central and peripheral action of ghrelin with respect to GI motility or, possibly, desensitization of the receptor. However, we believe it is more likely a reflection of the relatively high degree of variability, and limited dynamic range, of the SIT assay. Nevertheless, it is clear that centrally delivered synthetic GhrR agonists cause potent and lasting effects on both FI and GI motility.

The utility of GhrR agonism in a variety of models of GI pathology has been reported recently (Peeters, 2006; Sanger, 2008; Sanger and Alpers, 2008). Treatment with ghrelin has been shown to successfully ameliorate diabetic gastroparesis in both animal models of the disease (Qiu et al., 2008a) and in humans (Murray et al., 2005). Our results demonstrate that the efficacy of synthetic GhrR agonists is robust under a condition of pathological GI dysmotility, namely opiate-induced bowel dysfunction. Opioid mechanisms such as those elicited by morphine are well known to increase GI resting tone while decreasing propulsion contractions with the overall effect of delaying passage of contents through the GI tract (Greenwood-Van Meerveld et al., 2004). Oral treatment with the GhrR agonist, EX-1314, normalized morphine-induced dysmotility. Similar data have been obtained previously after parenteral dosing of a macrocyclic GhrR agonist (Venkova et al., 2007). These results show that ghrelin agonism is capable of over-riding the opiate-induced GI hypomotility mechanism and suggest a utility of ghrelin agonists for opiate-induced bowel dysfunction and possibly other forms of ileus as well.

In summary, we have demonstrated the upper GI prokinetic efficacy of small-molecule GhrR agonists of the di-methyl glycyl-O-benzyl-serine class dosed by either parenteral or oral routes. Furthermore, we provide evidence that GhrR agonist-induced feeding provides an indirect stimulation of increased fecal output. It is important to note that the actions of our agonists are highly GhrR-specific and probably involve central neuronal pathways. In addition, we show that stimulation of the GhrR increases upper GI motility in both a basal state and in a model of opiate-induced bowel dysfunction. It has been suggested recently that the dual action of GhrR agonists to stimulate both feeding and GI motility may provide a compelling therapeutic strategy for reversing the gastroparesis and cachexia associated with chemotherapy in cancer patients (Malik et al., 2008). Our data support the notion that small-molecule ghrelin agonists may offer a well-integrated therapeutic approach to the treatment of multiple GI hypomotility-related disorders.

Acknowledgments

We thank Dr. Kip Martha and the Elixir staff for support, advice, and encouragement and Dr. Gunraj Rakipovski (Rhoscience Inc.) for thoughtful comments during the course of this project.

References

releasing peptide-6 with the motilin receptor in the rabbit gastric antrum. J Phar-
macol Exp Ther 306:660–667.
De Smet B, Depoireire I, Moechars D, Swennen Q, Moreaux B, Cryns K, Tack J,
Buys E, Couillie B, and Peeters TL (2006) Energy homoeostasis and gastric empty-
stimulates gastric emptying but is without effect on acid secretion and gastric endo-
crine cells. Regul Pept 120:23–32.
Druce MH, Wren AM, Park AJ, Milton JE, Patterson M, Freet G, Ghatel MA, Small C,
and Bloom SR (2005) Ghrelin increases food intake in obese as well as lean
Fraser GL, Hoveyda HR, and Tannenbaum GS (2008) Pharmacological demarcation
of the growth hormone, gut motility and feeding effects of ghrelin using a novel
induces fasted motor activity of the gastrointestinal tract in conscious fed rats.
Fukuda H, Mizuta Y, Isomoto H, Takeshima F, Ohnita K, Ohka K, Omagari K,
stimulation of intrinsic neural pathways and capsaicin-sensitive afferent neurons
Greenwood-Van Meerveld B, Gardner CJ, Little PJ, Hicks GA, and Dehaven-
Smith, J, 7th ed, Institute of Laboratory Animal Resources, Commis-
sion on Life Sciences, National Research Council, Washington, DC.
Heightman TD, Scott JS, Longley M, Bordas V, Dean DK, Elliott R, Hutley G,
ghrelin (growth hormone secretagogue) receptor: I. Lead identification. Bioorg
Institute of Laboratory Animal Resources (1996) Guide for the Care and Use of
Laboratory Animals, 7th ed, Institute of Laboratory Animal Resources, Commiss-
on on Life Sciences, National Research Council, Washington, DC.
motor effects of peptide and non-peptide ghrelin agonists in mice in vivo and in
Leite-Moreira AF and Soares JB (2007) Physiological, pathological and potential
Levin F, Edholm T, Schmidt PT, Gysberts P, Jacobsson H, Degerbald M, Hoby C,
digestion and hunger in normal-weight humans. J Clin Endocrinol Metab 91:
3296–3302.
Malik NM, Moore GB, Kaur R, Liu YL, Wood SL, Morrow RW, Sanger GJ, and
receptors and increased plasma ghrelin in a model of cancer chemotherapy-
Mascolo N, Izzo AA, Ligresti A, Costagliola A, Pinto L, Fenu L, Yung Y, Shafranik A,
and Bloom SR, et al. (2005) Ghrelin increases food intake in obese as well as lean
M, and Kangawa K (2000) Ghrelin stimulates gastric acid secretion and motility in
Murray CD, Martin NM, Patterson M, Taylor SA, Ghatel MA, Kam M, Johnston
C, Bloom SR, and Emmanuel AV (2005) Ghrelin enhances gastric emptying in
diabetic gastroparesis: a double blind, placebo controlled, crossover study. Gut
54:1363–1369.
(2008a) Ghrelin improves delayed gastrointestinal transit in allaxon-induced dia-
betic mice. World J Gastroenterol 14:2572–2577.
Qiu WC, Wang ZG, Wang WG, Yan J, and Zheng Q (2008b) Gastric motility effects
of ghrelin and growth hormone releasing peptide 6 in diabetic mice with gastro-
Qiu WC, Wang ZG, Wang WG, Yan J, and Zheng Q (2008c) Therapeutic effects of
grelin and growth hormone releasing peptide 6 on gastroparesis in streptozo-
hormone secretagogue receptor expression in the cells of the stomach-projected
Sanger GJ (2008) Motilin, ghrelin and related neuropeptides as targets for the
disorders: translating science to clinical need. Neurogastroenter Mol Motil 20:
177–184.
Shimizu Y, Chang EC, Shafton AD, Ferens DM, Sanger GJ, Witherington J, and
Furness JB (2006) Evidence that stimulation of ghrelin receptors in the spinal cord
hormone release and appetite is mediated through the growth hormone secreta-
Tack J, Depoirtre I, Bischoops R, Peltorote C, Couillie B, Meulemans A, Janssens J,
and Peeters T (2006) Influence of ghrelin on interdigestive gastrointestinal motil-
Influence of ghrelin on gastric emptying and meal-related symptoms in idiopathic
pptide Y receptors are involved in 3rd ventricular ghrelin induced alleviation of
colonic transit time in conscious fed rats. BMC Gastroenterol 5:5.
(2005) Immature hypothalamic ghrelin is a potent neurotransmitter to reverse gastric
postprandial ileus in rats. Am J Physiol Gastrointest Liver Physiol 392:G948–
G952.
netic effects of a new ghrelin receptor agonist TFP-101 in a rat model of postprap-
Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillon WS, Ghatel
MA, and Bloom SR (2001) Ghrelin enhances appetite and increases food intake in
Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taberi S, Kennedy AR,
ghrelin stimulates food intake and growth hormone secretion. Endocrinology
141:4375–4378.
Xu L, Depoireire I, Tomassetto C, Zandecki M, Tang M, and Timmersmans JP, and
Peeters TL (2005) Evidence for the presence of motilin, ghrelin, and the motilin
ghrelin receptor in neurons of the myenteric plexus. Regul Pept 124:119–125.

Address correspondence to: Dr. Brad J. Geddes, Elixir Pharmaceuticals, Inc.,
12 Emily Street, Cambridge, MA 02139. E-mail: bgeddes@elixirpharm.com