

Prokinetic Effects of a New Ghrelin Receptor Agonist TZP-101 in a Rat Model of Postoperative Ileus

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Abstract Postoperative ileus (POI) is a major cause of postoperative complications and prolonged hospitalization. Ghrelin, which is the endogenous ligand for the growth hormone secretagogue receptor, has been found to stimulate gastric motility and accelerate gastric emptying. The present study investigates whether TZP-101 (0.03–1 mg/kg i.v.), a synthetic ghrelin-receptor agonist, could improve gastrointestinal transit in rats with POI. Since the main factors for the development of POI are the surgical manipulation and the gastrointestinal effects of opioid-receptor agonists used for pain management, the effect of TZP-101 was investigated in rats subjected to surgery, to morphine treatment (3 mg/kg s.c.), or to a combination of both. The results showed that TZP-101 is equally effective against the delayed gastrointestinal transit induced by surgery, by morphine, or by the combination of both interventions. The prokinetic action of TZP-101 was more pronounced in the stomach compared to the small intestine.

Keywords Ghrelin-receptor agonist · Postoperative ileus · Gastrointestinal transit

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Introduction

Ghrelin is a 28-amino acid, octanoylated gut peptide hormone and an endogenous ligand of the growth hormone secretagogue receptor (GHS-R1a, a G-protein coupled receptor) [1]. Ghrelin is most widely recognized for its activity to stimulate growth hormone release and increase food intake [2, 3]. Furthermore, there is growing evidence that ghrelin also stimulates gastric emptying and intestinal motility [4]. Ghrelin is predominantly produced by endocrine cells of the oxyntic mucosa of the stomach [5, 6], where it acts locally on receptors expressed on myenteric neurons [7, 8]. Also, ghrelin has been shown to elicit prokinetic activity via activation of vagal afferents [9, 10] and central pathways [11, 12]. In animal models of dysmotility, ghrelin corrected impaired motility in the upper gastrointestinal (GI) tract caused by postoperative ileus (POI), septic ileus, or cisplatin treatment [13–15]. In the clinic, ghrelin has been shown to accelerate gastric emptying in volunteers [16] and in patients with diabetic or idiopathic gastroparesis [17–19]. Taken together, these findings suggest that ghrelin agonists may represent a novel class of gastroprokinetic agents for the treatment of GI dysmotility.

POI is the transient impairment of GI function developing as a consequence of abdominal surgery [20]. Opioid drugs administered for postsurgical pain management are known to exacerbate the severity of POI [21] due to the activation of opioid receptors and consequent inhibition of enteric reflexes and suppression of GI motility [22]. Since postsurgical GI dysmotility is a major factor contributing to patient morbidity and prolonging hospital stay, the development of new pharmacological strategies to accelerate the recovery from POI are clinically important [23].

TZP-101 is a small-molecule ghrelin-receptor agonist with superior bioavailability to the ghrelin peptide. TZP-101

has previously been reported to increase gastric emptying and small intestinal transit in rats without altering growth hormone release [24]. In the present study, the prokinetic effect of TZP-101 was investigated in a rat model of POI. To mimic the situation in patients with abdominal surgery, rats were subjected to morphine treatment followed by a surgical procedure known as “running of the bowel” to induce POI [25]. The effect of TZP-101 was studied on GI transit following surgical manipulation, morphine treatment, or the combination of both interventions.

Methods

Animals

Male Sprague-Dawley rats (250–350 g) were used in the experiments. The animals were ordered from Charles River Laboratories (Wilmington, MA) with a polyurethane catheter implanted into the right jugular vein. The exterior end of the catheter was secured to the dorsal neck area and used for i.v. injection. Animals were housed one per cage under standard conditions (21–22°C, 12-hr light/dark cycle, controlled humidity), with water and rat chow available *ad libitum*. Prior to initiating the experiments the rats were allowed 1 week of acclimation to the animal facility. Catheters were maintained by flushing with heparinized dextrose solution once every 3–4 days but not on the day of the experiment. The experimental protocols and number of animals were approved by the Animal Care and Use Committees at the VA Medical Center and the University of Oklahoma Health Sciences Center, Oklahoma City.

Induction of postoperative ileus

Prior to the experiments, the rats were fasted for 18–20 hr with free access to water. POI was induced by a surgical procedure described as “running of the bowel” [25]. Specifically, the rats were anesthetized with isoflurane (2%–3%) inhalation, the abdomen was shaved and disinfected, and a midline incision was made. The small intestine and the cecum were exteriorized and inspected for 5 min using cotton applicators soaked in sterile saline. After completing the inspection, the intestines were covered with gauze soaked in saline and the abdomen was left open for a total of 10 min. The viscera were then placed back into the abdomen and the incision was closed with silk sutures. The procedure lasted 20–25 min, then an additional period of 15 min was allowed for the animals to recover completely from the anesthesia before being treated with the drug or the vehicle.

Evaluation of GI transit

Conscious rats received an intragastric gavage of 1.5 ml of ^{99m}Tc in 1.5% methylcellulose solution in distilled water. ^{99m}Tc radioactivity was adjusted to approximately 100,000 cpm. After receiving the radioactive meal, the animals were placed in a wire-bottom cage without access to food and water to allow normal GI transit of the intragastric content. All rats were euthanized by CO_2 inhalation 15 min after receiving the meal. After the abdomen was opened, the stomach was clamped using a single silk ligature at the esophageal junction and two parallel ligatures between the pyloric junction and the duodenum. The stomach with the whole length of the small intestine attached was then isolated and 10-cm sections of the intestine were separated by ligatures starting from the pyloric junction. The stomach and the ligated intestinal sections were carefully excised and placed in separate test tubes. Radioactivity remaining in the stomach and in the intestinal segments, arranged in proximal to distal order, was measured using a gamma-counter. The data (cpm) are expressed as a percentage of the total recovered radioactivity. Gastric emptying was measured by the percentage of total recovered radioactivity that entered the intestine 15 min after the intragastric infusion. The small intestinal transit was assessed by the geometric center calculated as a function of the amount of intragastric content transported to each segment along the intestine. Calculations were made according to the method of Miller and coworkers [26]: $\text{geometric center} = \frac{\sum(\% \text{ of total radioactivity per segment} \times \text{number of segment})}{100}$. Transit was characterized also by the maximal distance (cm) reached by the head of the meal along the length of the small intestine.

Experimental protocol

Three series of experiments were performed to investigate the effect of TZP-101 on the GI transit of a semiliquid, noncaloric meal. The models of POI including surgery and/or morphine treatment were selected to mimic the clinical situation in patients with abdominal surgery receiving opioids for postsurgical analgesia. In the first series of experiments, the rats were subjected to “running of the bowel” surgery to induce POI. In the second series, the rats received subcutaneous injections of morphine (3 mg/kg s.c.). The dose of morphine was selected based on the results of a pilot study that demonstrated a significant delay in gastric emptying and small intestinal transit measured for a period of 60–75 min after morphine administration (data not shown). In the third series of experiments, the rats were pretreated with morphine (3 mg/kg s.c.) for 15 min and were then subjected to “running of the bowel” surgery. The dose-response

Table 1 Effect of “running of the bowel” surgery and morphine treatment on the gastrointestinal transit in rats

Rat model	Gastric emptying (%)	Geometric center	Distance (cm)
Naïve ($n = 8$)	69.7 ± 4.7	4.92 ± 0.53	75.0 ± 3.3
Surgery ($n = 10$)	$26.3 \pm 3.6^*$	$1.57 \pm 0.15^*$	$31.0 \pm 4.9^*$
Morphine ($n = 7$)	$33.0 \pm 4.4^*$	$1.79 \pm 0.22^*$	$55.7 \pm 6.8^*$
Morphine + surgery ($n = 7$)	$29.3 \pm 5.5^*$	$1.28 \pm 0.08^*$	$38.0 \pm 4.5^*$

Note. Data are the mean \pm SE of 7–10 experiments in each group. Statistical significance was assessed by one-way ANOVA followed by Bonferroni’s MCT. * $p < 0.05$ compared to naïve.

effect of increasing doses of TZP-101 (0.03–3 mg/kg bolus infusion via the jugular catheter) on the delayed gastric emptying and GI transit was investigated in these three rodent models. TZP-101 was administered within 1–2 min prior to the intragastric gavage of the radiolabeled methylcellulose meal.

Test and control articles

The test compound, TZP-101, was kindly provided by Tranzyme Pharma (Sherbrooke, QC, Canada). A stock solution of 4 mg/ml TZP-101 in 9% (w/v) 2-hydroxypropyl- β -cyclodextrin was prepared in sterile saline. The stock solution was kept at 4°C and used to prepare fresh dilutions for each experiment for 2–3 consecutive days. 2-Hydroxypropyl- β -cyclodextrin used in vehicle control experiments was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in sterile saline. Morphine sulfate was obtained from Baxter Healthcare Corp. (Dearfield, IL) and dissolved in sterile saline.

Binding assays

TZP-101 was tested in radioligand binding assays at the human ghrelin receptor (GHS-R1a) and the human μ , δ , and κ opioid receptors, each individually and stably expressed in HEK293 cells. In the case of the opioid receptors, TZP-101 was tested in duplicate samples at a single concentration of 10 μ M relative to total and nonspecific binding measures. In the case of GHS-R1a, an 11-point competition binding curve was established in half-log increments over a range of concentrations from 0.0003 to 10 μ M. [¹²⁵I]Ghrelin (human) was used as the radioligand for displacement ($K_d = 0.01$ nM; test concentration, 0.007 nM) and unlabeled ghrelin (1 μ M) was used to determine nonspecific binding. In all cases, the binding reaction was allowed 60 min to reach equilibrium prior to filtration to separate bound and free fractions and subsequent measurement of radioactivity by liquid scintillation counter. Data were analyzed by GraphPad Prism using a variable slope nonlinear regression analysis to determine the IC₅₀ values for TZP-101.

Data analysis and statistics

Gastric emptying, the geometric center, and the maximal distance reached by the head of the meal are expressed as means \pm SE of five to eight subjects in each group. The effect of TZP-101 was evaluated by the differences between the mean values obtained for the TZP-101-treated groups and the vehicle-treated group. Statistical significance was assessed using one-way ANOVA followed by Dunnett’s test (for comparison to a control group) or by Bonferroni’s multiple-comparison test, where $P < 0.05$ was considered significant. The activity of TZP-101 to normalize gastric emptying and small intestinal transit in the models of delayed GI transit was expressed in terms of gastric emptying, geometric center, and distance to the most distal segment reached by the meal as a percentage of the respective mean values in naïve rats with normal GI transit.

Results

TZP-101 was well tolerated by the rats when administered as a bolus i.v. infusion in a volume of 1 ml. The animals showed no changes in behavior following TZP-101 treatment at doses ranging from 0.03 to 3 mg/kg.

POI induced by surgery

The effect of TZP-101 was first measured in rats with POI in the absence of morphine. As expected, “running of the bowel” induced a postsurgical delay in gastric emptying and small intestinal transit compared to naïve rats without surgery (Table 1). When the animals were treated with TZP-101 (0.03–1 mg/kg) gastric emptying showed a statistically significant, dose-dependent increase to 96.9% of the values in naïve animals (Fig. 1A). Gastric emptying observed in POI rats treated with 0.3 or 1 mg/kg TZP-101 did not differ significantly from that measured in naïve rats. The geometric center (Fig. 1B) and the distance reached by the head of the meal (Fig. 1C) were also significantly increased by TZP-101, up to a maximum of 60.9% and 62.8%, respectively, in comparison to control values in naïve rats.

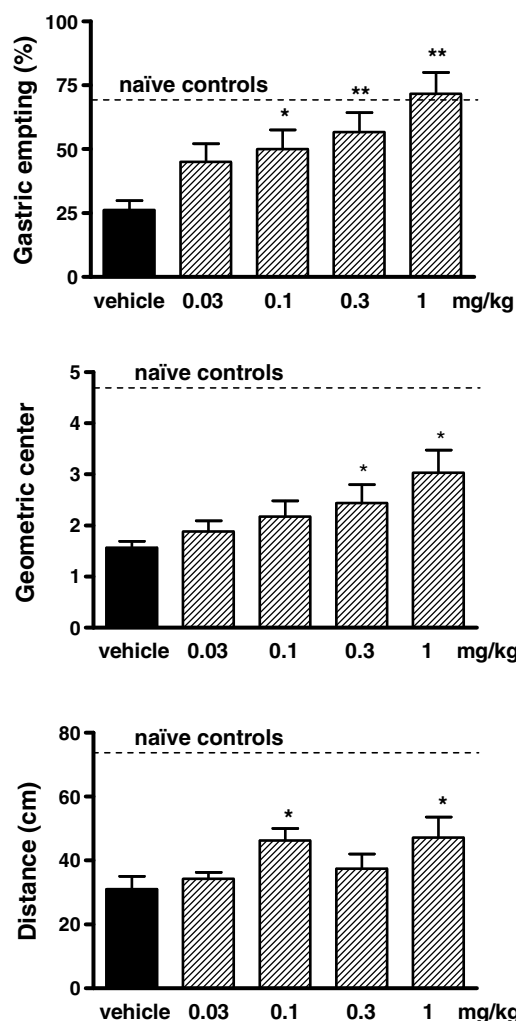


Fig. 1 Effects of TZP-101 in a rat model of delayed GI transit induced by abdominal surgery. TZP-101 administered at doses of 0.03–1 mg/kg (i.v.) induced a dose-dependent increase in gastric emptying (upper panel), geometric center (middle panel), and distance reached by the head of the meal (lower panel). Data are mean \pm SE for seven or eight experiments in each group. Statistical significance was assessed by one way ANOVA followed by Dunnett's test for multiple comparisons. * $P < 0.05$ and ** $P < 0.01$ compared to vehicle

Morphine-induced delay in GI transit

Opioids used for the treatment of pain during the perioperative period cause a pronounced delay in gastric emptying and intestinal transit. Pilot studies demonstrated that a 3 mg/kg dose of morphine (administered s.c., 60 min prior to meal) induced a significant delay in gastric emptying and reduced small intestinal transit without otherwise affecting the behavior of the animal (Table 1). Thus, a 3 mg/kg dose of morphine was selected for the present set of experiments, whereupon TZP-101 was tested at doses of 0.3 and 1 mg/kg. In morphine-treated rats, TZP-101 (1 mg/kg) significantly accelerated gastric emptying (Fig. 2A) and increased the ge-

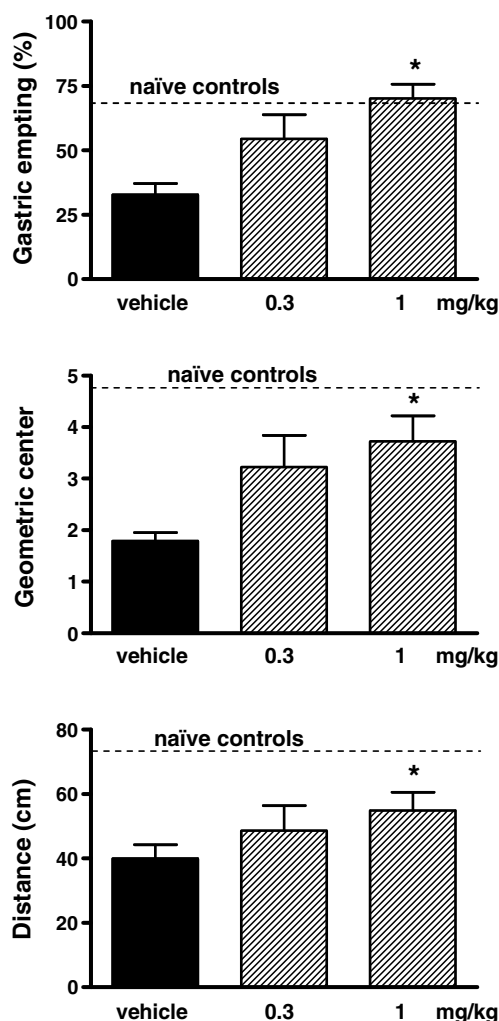


Fig. 2 Effects of TZP-101 in a rat model of delayed GI transit-induced morphine treatment (3 mg/kg, s.c.). TZP-101 administered at doses of 0.3 or 1 mg/kg (i.v.) induced a dose-dependent increase in gastric emptying (upper panel), geometric center (middle panel), and distance reached by the head of the meal (lower panel). Data are mean \pm SE for six or seven experiments in each group. Statistical significance was assessed by one-way ANOVA followed by Dunnett's test for multiple comparisons. * $P < 0.05$ compared to vehicle

ometric center (Fig. 2B) and the distance reached by the meal (Fig. 2C). When the results were compared to the data in naïve animals there was a complete recovery of the morphine-induced delay in gastric emptying, to 101.1%. However, the maximal recovery of the geometric center and distance to the head of the meal reached 75.8% and 73.3%, respectively.

POI induced by a combination of morphine and surgery

In this series of experiments, the rats were treated with 3 mg/kg morphine for 15 min prior to the "running of the

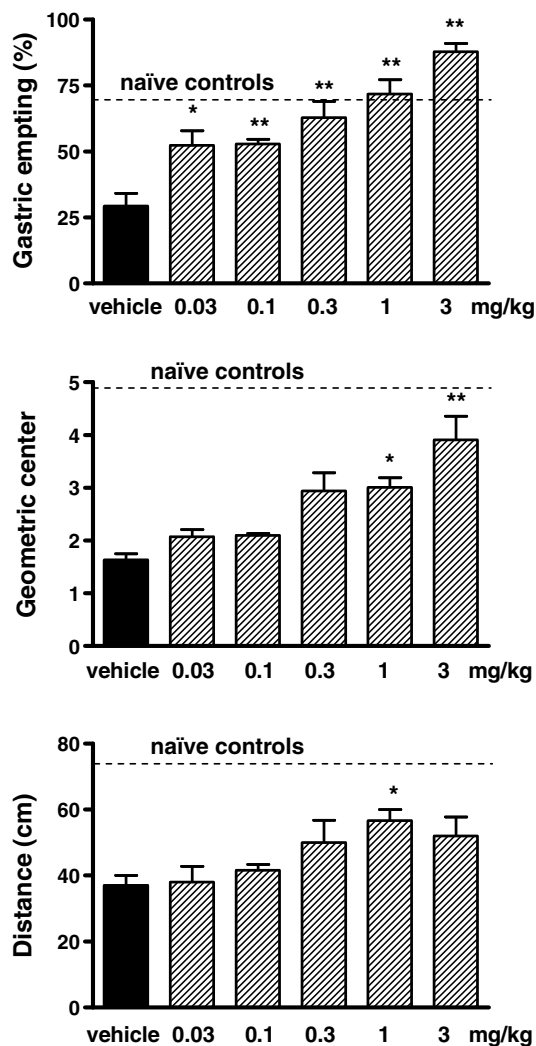


Fig. 3 Effects of TZP-101 in a model of delayed GI transit induced by abdominal surgery in rats pretreated with morphine (3 mg/kg, s.c.). TZP-101 administered at doses of 0.03–3 mg/kg (i.v.) induced a dose-dependent increase in gastric emptying (upper panel), geometric center (middle panel), and distance reached by the head of the meal (lower panel). Data are mean \pm SE for seven to nine experiments in each group. Statistical significance was assessed by one-way ANOVA followed by Dunnett's test for multiple comparisons. * $P < 0.05$ and ** $P < 0.01$ compared to vehicle

bowel" surgery. The procedures resulted in a significant decrease in gastric emptying and a delay in small intestinal transit compared to naïve rats (Table 1). TZP-101 administered to rats with POI at doses of 0.03–3 mg/kg induced a dose-dependent increase in gastric emptying up to 126% of control values in naïve rats at the high dose of 3 mg/kg. In this experiment, the increase in gastric emptying was statistically significant at all doses (Fig. 3A). The acceleration of gastric emptying was associated with an increase in the geometric center (Fig. 3B) and the distance along the small intestine reached by the radiolabeled meal (Fig. 3C), although the latter effects reached significance only at doses of 1–3 mg/kg.

Table 2 TZP-101 Binding Selectivity for GHS-R1a in comparison to μ , δ , and κ opioid receptors

Human receptor clone	% inhibition of control specific binding at 10 μ M	Actual/projected ^a IC ₅₀ (μ M)
GHS-R1a	97 \pm 3	0.031 \pm 0.003
μ opioid	23 \pm 6	> 10
δ opioid	13 \pm 1	> 10
κ opioid	60 \pm 7	8.3 \pm 0.7

^aIC₅₀ values are not calculated when % inhibition <50% at 10 μ M.

Data are presented as mean \pm SE.

TZP-101 treatment induced a 75.8% recovery of the geometric center and a 73.3% recovery of the distance reached by the head of the meal compared to those in naïve rats.

TZP-101 binding selectivity study

Competition binding studies were performed in order to determine whether the reversal of morphine-induced delay in gastric emptying and GI transit could be due to a nonselective interaction of TZP-101 at opioid receptors. The pharmacological actions of morphine are modulated predominantly by the μ opioid receptor, with minor, nonselective signaling through the δ and κ opioid subtypes [27]. TZP-101 demonstrated >200-fold selectivity in binding affinity for GHS-R1a in comparison to any of the opioid receptors based on the comparative ratios of IC₅₀ values (Table 2).

Discussion

The present study demonstrates that selective activation of ghrelin receptors by systemic administration of the ghrelin receptor agonist TZP-101 ameliorates the symptoms of POI in morphine-treated rats subjected to abdominal surgery. TZP-101 is a synthetic small molecule acting as a selective ghrelin agonist with gastroprokinetic activity [24]. In the current study, TZP-101 normalized the delayed gastric emptying in a rat model of POI similarly to the response observed for ghrelin and other ghrelin receptor agonists [13, 28]. TZP-101 also induced a significant recovery in small intestinal motility in the current study, albeit with a lower potency than observed for its effects on gastric emptying similar to the activity reported for ghrelin peptide [13]. Furthermore, TZP-101 normalized the delayed gastric emptying and reduced the small intestinal transit caused by morphine. The > 200-fold selectivity of TZP-101 for the ghrelin receptor (GHS-R1a) versus opioid receptors in binding studies indicate that this effect is not due to nonselective, direct antagonism by TZP-101 of opioid receptors.

In the current study, abdominal surgery was performed in morphine-pretreated rats in order to mimic the clinical situation for POI. Although gastric emptying and small intestinal transit were suppressed significantly by either abdominal surgery or morphine treatment, surprisingly, the combination of morphine treatment and surgery did not lead to any further measurable degree of inhibition of GI transit as that of each individual intervention, a finding consistent with a previous report [28]. Thus, it is likely that 70%–75% meal retention (corresponding to 25%–30% gastric emptying; Table 1) is the maximum delay of gastric emptying detectable in this model. TZP-101 showed similar efficacy and potency in causing a complete reversal of the delay in gastric emptying and accelerating small intestinal transit in the three models presented in the current study. This finding is in contrast to another small-molecule ghrelin agonist, RC-1139, where potency decreased when POI and morphine treatment were combined [28]. The equivalent efficacy and potency of TZP-101 in either the POI model or the morphine model, or both models combined, suggest that TZP-101 activity is mediated by a common, overriding pathway mediating prokinetic activity.

Although the precise mechanism of POI is not completely understood, it is known that the impairment of GI motility induced by surgery is the result of multiple causes including stimulation of afferent neurons and consequent activation of noradrenergic, nonadrenergic–noncholinergic (NANC) [29] and tachykinergic [30] neuronal pathways as well as the induction of an intestinal inflammatory response [31–33]. Recent studies suggest that ghrelin agonists may overcome ileus via a direct anti-inflammatory activity in the gastric mucosa [34] and by stimulating excitatory motility pathways, including direct modulation of tachykinergic neurons [35]. Only the motility effects of TZP-101 were examined in the present study.

There appears to be more than one mode of action whereby ghrelin agonists such as TZP-101 may elicit prokinetic activity, and the dominance of each mode of action seems to differ depending on the region of the GI tract involved. Thus, the effect of ghrelin agonists to increase gastric emptying is likely the product of multiple pathways, including direct effects on local receptors on cholinergic, enteric neurons [7, 35, 36], activation of vagal afferents [37, 38], perhaps via direct modulation of tachykinergic neurons [39], and stimulation of central pathways [38]. The role of vagal afferent neurons in mediating the action of peripheral ghrelin is supported by the finding of ghrelin-receptor mRNA on vagal afferent neurons using *in situ* hybridization [40] and in neuronal cell bodies of stomach-projected nerves in the vagal nodose ganglion [41]. The activity of these receptors has been demonstrated by electrophysiological experiments showing that intravenous administration of ghrelin suppresses the discharge of gastric vagal afferents in the rat [37]. However, activity at local

enteric neurons is also implicated since it has been shown that ghrelin is still able to induce fasted motor activity in the stomach and the duodenum in vagotomized rats [38]. Furthermore, experiments in isolated gastric fundus muscle strips revealed that ghrelin potentiates the contractions induced by electrical field stimulation of enteric nerves without any direct effect on the gastric muscle [35]. Ghrelin receptors located on local cholinergic nerves have also been shown to play a role in the acceleration of gastric emptying in mice *in vivo* [36] as well as in rat duodenum, where ghrelin elicited an atropine-sensitive increase in the frequency of interdigestive migrating myoelectric complex (MMC) [42]. The actions of ghrelin agonists in regions of the GI tract apart from the stomach have been characterized less extensively. Thus, mechanistic studies with ghrelin on small intestine motility have solely implicated activation of cholinergic neurons in the myenteric plexus [8, 42]. In contrast, the effect of ghrelin on colonic motility appears to be centrally mediated via sites in the paraventricular nucleus of the hypothalamus [11] or the lumbosacral region of the spinal cord [12]. Taken together, a ghrelin agonist with both central and peripheral modes of action may be optimal for the treatment of POI since the severity of POI stasis is greatest in the stomach and colon [21].

In clinical studies, the duration of POI following colorectal surgery is positively correlated with the amount of blood loss, surgery time, and postoperative opioid dosage [43]. A retrospective study of POI patients also identified use of opioid-based patient-controlled analgesia as a key risk factor [44]. Although the analgesic effects of opioids such as morphine are predominantly mediated by μ opioid receptors in the central nervous system, the action of morphine to delay GI transit involves both central and peripheral pathways [45–47]. These peripheral pathways may be selectively blocked by peripherally restricted opioid antagonists such as alvimopan [23, 48] and methylnaltrexone [49], agents currently in clinical development for the treatment of POI. In contrast to TZP-101, these opioid antagonists do not have direct prokinetic effects [22] and they are most effective when dosed prior to surgery in a rat model of POI [48, 50].

In conclusion, our results support the role played by ghrelin in the regulation of gastric and small intestinal motility, indicating that selective activation of ghrelin receptors may overcome the motility dysfunction associated with POI. Moreover, this study showed that TZP-101, a small-molecule ghrelin-receptor agonist, is equally effective against the delay in GI transit induced by manipulation of the bowel during abdominal surgery, by morphine, or by the combination of both interventions in the rat. The prokinetic action of TZP-101 was more pronounced in the stomach compared to the small intestine, a finding consistent with the reports in the literature for ghrelin and alternate ghrelin agonists. In summary, these results imply that TZP-101 may

represent a new therapeutic approach for the treatment of gastric and small intestinal dysmotility in patients with POI.

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