Synergistic Effect of 5-Hydroxytryptamine 3 and Neurokinin 1 Receptor Antagonism in Rodent Models of Somatic and Visceral Pain

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ABSTRACT
Synergistic activity has been observed between serotonergic 5-hydroxytryptamine 3 (5-HT3) and tachykinergic neurokinin 1 (NK1) receptor–mediated responses. This study investigated the efficacy of a 5-HT3 antagonist, palonosetron, and a NK1 antagonist, netupitant, alone or in combination in rodent models of somatic and visceral colonic hypersensitivity. In a rat model of experimental neuropathic pain, somatic hypersensitivity was quantified by the number of ipsilateral paw withdrawals to a von Frey filament (6 g). Electrophysiologic responses were recorded in the dorsal horn neurons after mechanical or thermal stimuli. Acute colonic hypersensitivity was induced experimentally in rats by infusing dilute acetic acid (0.6%) directly into the colon. Colonic sensitivity was assessed by a visceromotor behavioral response quantified as the number of abdominal contractions in response to graded isobaric pressures (0–60 mm Hg) of colorectal distension. Palonosetron or netupitant was administered alone or in combination via oral gavage. When dosed alone, both significantly reduced somatic sensitivity, decreased the evoked response of spinal dorsal horn neurons to mechanical or thermal stimulation, and caused significant (P < 0.05) inhibition of colonic hypersensitivity in a dose-dependent manner. The combined administration of palonosetron and netupitant at doses that were ineffective alone significantly reduced both somatic and visceral sensitivity and decreased the evoked response of spinal dorsal horn neurons to mechanical or thermal stimulation. In summary, the combination of palonosetron with a NK1 receptor antagonist showed synergistic analgesic activity in rodent models of somatic and visceral hypersensitivity, and may prove to be a useful therapeutic approach to treat pain associated with irritable bowel syndrome.

Introduction
Chronic abdominal pain is the hallmark feature of irritable bowel syndrome (IBS) and the main reason patients with IBS seek medical attention (Poitras et al., 2002; Zhou et al., 2010). Many patients with IBS also suffer from fibromyalgia with chronic somatic pain (Chang, 2005); however, the mechanisms of impaired antinoceception in IBS are poorly understood. Currently, few therapies exist to treat either chronic abdominal or somatic pain in patients with IBS, and available treatments are limited by poor side-effect profiles and inadequate efficacy. Thus, there is an urgent need to treat chronic pain in patients with IBS with novel therapies devoid of life-threatening side effects. Neuropathic pain is also a major clinical problem, with a lack of efficacy of common analgesics. In many cases, single-agent therapy is not sufficient. As a result, combination therapy is often used, and the issues behind this type of approach were recently reviewed (Gilron et al., 2013).

Serotonin has been implicated as a key neurotransmitter in the control of nociceptive responses, with sites of action located in both the peripheral and central nervous systems. Depletion of spinal 5-hydroxytryptamine (5-HT) reduces behavioral mechanical hypersensitivity after nerve injury and reduces the formalin response, suggesting that there is an endogenous 5-HT facilitation (Green et al., 2000). Evidence indicates that 5-HT3 receptors mediate a descending facilitatory influence on spinal cord activity, a constituent drive that is particularly prominent on mechanically and chemically evoked activity (Green et al., 2000). This activity is enhanced after peripheral nerve injury, spinal cord injury, and intense chemical

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; AA, acetic acid; CRD, colorectal distension; IBS, irritable bowel syndrome; NK1, neurokinin 1; PKC, protein kinase C; SNL, spinal nerve ligation; SP, substance P.
sympathetic sensory nerves is mediated by peripheral release of substance P
(Wood et al., 2008). In this study, we examined whether 5-HT3 receptor antagonism was
shown to potentiate 5-HT3 receptor responses, as seen in postganglionic sympathetic
ergic responses (Li et al., 2003). More recently, synergistic activity was observed between
5-HT3 and tachykinergic NK1 responses in vitro (Minami et al., 2001). In this study, we examined whether 5-HT3 activation and NK1 responses could interact in vivo, resulting in a synergism of efficacy in experimental models of somatic and visceral pain.

In the first series of experiments, the effects of 5-HT3 and NK1 antagonism were investigated using palonosetron, a second-generation 5-HT3 antagonist, and netupitant, a potent and selective NK1 antagonist, evaluated alone and in combination in a rat model of experimental neuropathic pain established by ligature of spinal nerves. Electrophysiologic responses were recorded in the dorsal horn neurons after mechanical and thermal stimuli. In a second series of experiments, the activity of the two compounds alone and combined was studied in a rodent model of visceral colonic hypersensitivity. A preliminary report of some of the observations was previously presented in abstract form at Digestive Disease Week in San Diego (Pietra et al., 2012).

Materials and Methods
Experimental Series 1: Assessment of Behavioral and Electrophysiologic Responses to Chronic Somatic Pain

Animals. Male Sprague-Dawley rats (330–480 g at time of colonic sensitivity assessment) were purchased from Charles River Laboratories. Rats were housed two per cage within the University of Oklahoma Health Sciences Center (OUHSC) Department of Comparative Medicine's animal facility under controlled temperature and humidity. All animals had free access to food and water and were acclimated to facility housing for a minimum of 1 week before experiment. A total of 85 rats were used to complete this study. The experimental protocol was approved by the OUHSC Institutional Animal Care and Use Committee (Animal Protocol 10-077). Upon arrival, all animals were acclimated to the animal facility for a minimum of 3 weeks. To further acclimate and minimize experimental stress, the rats were brought to the laboratory for an additional week to acclimate to the laboratory environment and animal handlers.

Induction of Acute Visceral Hypersensitivity. Visceral hypersensitivity was induced by infusing dilute (1.5 ml at 0.6%) acetic acid (AA) into the rat colon via a catheter (PE-90 tubing; BD Intramedic, Sparks, MD) inserted via the anus to the level of the midcolon. Within...
60 minutes, colonic hypersensitivity was evident. Visceromotor responses to colorectal distension (CRD) were measured by counting the number of abdominal contractions in response to increasing levels of CRD (0–60 mm Hg). On the day of the colonic sensitivity assessment, a minor surgical procedure was performed to attach a strain gauge force transducer onto the abdominal oblique muscle and was connected via an adapter cable to a Grass Model 7 Polygraph (Grass Technologies, Warwick, RI). The cable was connected to a Model 7P1 low-level DC preamp (Marantz, Mahwah, NJ). The preamp was set at 0.02 mV/cm sensitivity and was connected to a Model 7DA DC driver amp (Marantz) with sensitivity set at 5.5 A. A 5-cm latex balloon was inserted into the distal colon. The balloon cannula was connected to a Distender Series IIR preamp (Marantz, Mahwah, NJ). The preamp was set at 0.02 mV/cm sensitivity and was connected to a Model 7D Polygraph (Grass Technologies, Warwick, RI). The cable was connected to a Model 7P1 low-level DC power cord to a Grass Model 7 Polygraph (Grass Technologies, Warwick, RI). The cable was set at 0.02 mV/cm sensitivity and was connected to a Model 7DA DC driver amp (Marantz) with sensitivity set at 5.5 A. A 5-cm latex balloon was inserted into the distal colon. The balloon cannula was connected to a Distender Series IIR preamp (Marantz, Mahwah, NJ). The preamp was set at 0.02 mV/cm sensitivity and was connected to a Model 7D Polygraph (Grass Technologies, Warwick, RI). The cable was set at 0.02 mV/cm sensitivity and was connected to a Model 7DA DC driver amp (Marantz) with sensitivity set at 5.5 A. A 5-cm latex balloon was inserted into the distal colon. The balloon cannula was connected to a Distender Series IIR preamp (Marantz, Mahwah, NJ). The preamp was set at 0.02 mV/cm sensitivity and was connected to a Model 7D Polygraph (Grass Technologies, Warwick, RI).

**Results**

Experimental Series 1a: Rodent Model of Somatic Hypersensitivity. Palonosetron when administered alone, at doses of 0.3 and 3 mg/kg s.c., significantly reduced somatic hypersensitivity as demonstrated by a reduction in the number of ipsilateral paw withdrawals to a von Frey filament (6g). In this assay, a nonsignificant inhibitory effect of palonosetron was determined to be 0.03 mg/kg s.c. In the same experimental assay, we found that netupitant when dosed alone (1 and 10 mg/kg s.c.) significantly reduced somatic hypersensitivity, whereas no significant effect was observed at a dose of 0.1 mg/kg s.c. Combined administration of these ineffective doses of palonosetron (0.03 mg/kg s.c.) and netupitant (0.1 mg/kg s.c.) significantly reduced somatic sensitivity compared with vehicle controls (Fig. 1).

Experimental Series 1b: Spinal Horn Responses to Mechanical and Thermal Stimulation. In this series, palonosetron and netupitant were administered alone and in combination. For each experimental condition, electrophysiologic tests of mechanical and thermal stimulations were carried out at 10, 30, and 50 minutes postdose. The maximal drug effect during this time period was calculated and used to represent the overall drug effect. Palonosetron and netupitant administered alone dose dependently decreased the spinal dorsal horn neuronal response to mechanical (Fig. 2) and thermal (Fig. 3) stimuli in an SNL model of pain. Palonosetron at a dose of 0.3 mg/kg significantly reduced the number of action potentials fired by spinal dorsal horn neurons at von Frey 6g. A dose of 3.0 mg/kg significantly decreased the evoked neuronal response at von Frey 8g, 26g, and 60g (Fig. 2A). Netupitant significantly
decreased the evoked response to von Frey 60g at doses of 0.1, 1, and 10 mg/kg compared with vehicle. A dose of 10 mg/kg netupitant also significantly decreased the number of action potentials fired by spinal dorsal horn neurons in response to von Frey 26g (Fig. 2B). In the thermal stimulation experiments, palonosetron (0.3 and 3 mg/kg s.c.) significantly decreased the thermally evoked responses of spinal dorsal horn neurons, although no significant effect was observed at a dose of 0.03 mg/kg s.c. (Fig. 3A). Netupitant (0.1, 1, and 10 mg/kg s.c.) significantly decreased the thermally evoked responses of spinal dorsal horn neurons. In both the mechanical and thermal (40 and 45°C) stimulation tests, the ineffective dose of either palonosetron or netupitant was observed but it was not possible to demonstrate an ineffective dose of netupitant at a thermal stimulus of 48°C (Fig. 3B). The combined administration of palonosetron and netupitant, at doses shown to be ineffective individually, significantly decreased the evoked response of spinal dorsal horn neurons to von Frey filaments (Fig. 2C) or to a thermal stimulus (Fig. 3C) compared with vehicle controls.

**Experimental Series 2: Rodent Model of AA-Induced Visceral Hypersensitivity.** Intracolonic infusion of dilute AA resulted in significant colonic hypersensitivity in response
to CRD, quantified as the number of abdominal muscle contractions, at distension pressures of 0, 40, and 60 mm Hg compared with normal saline controls (data not shown). Palonosetron (0.01 and 0.1 mg/kg p.o.) or netupitant (0.01 and 1.0 mg/kg p.o.) dosed alone via oral gavage (p.o.) resulted in significant inhibition of AA-induced colonic hypersensitivity; no effect was observed with a 0.001 mg/kg p.o. dose of either palonosetron or netupitant (Fig. 4, A and B). However, when dosed in combination at ineffective doses palonosetron and netupitant caused a significant inhibition of colonic hypersensitivity compared with the vehicle control (Fig. 4C).

**Discussion**

In this study, the effect of either a selective 5-HT₃ receptor antagonist or a selective NK₁ receptor antagonist, administered alone or in combination, was investigated in a series of experimental models of somatic and visceral pain in vivo. Our experiments revealed that low doses of either palonosetron or netupitant alone had no significant inhibitory effect in SNL animals with respect to behavioral responses of deep dorsal horn neurons to a range of stimuli. However, coadministration of both resulted in robust and consistent inhibition of behavioral and electrophysiologic indices of pain that generally peaked within the first hour after administration of the compounds. Similarly, in a rodent model of visceral colonic hypersensitivity, we found that when administered together at doses shown to be ineffective, palonosetron and netupitant resulted in significant inhibition of colonic hypersensitivity. Taken together, these data suggest that the combination of 5-HT₃ and NK₁ receptor antagonism blocks the spinal origins and the descending endpoint of the pronociceptive loop through synergistic effects (Stacher, 2002).

A key factor in this study is the receptor selectivity of the chosen 5-HT₃ and NK₁ receptor antagonists. Palonosetron is an antiemetic drug that, unlike first-generation 5-HT₃ receptor antagonists, has been found to be effective in preventing both acute and delayed chemotherapy-induced nausea and vomiting (Gralla et al., 2003; Rubenstein, 2004; Siddiqui and Scott, 2004; Ho and Gan, 2006). In this study, the first step for the in vivo pharmacological characterization of palonosetron was the investigation of the compound in an assay of somatic pain induced by nerve injury in a rodent model. In this investigation, we performed SNL surgery to induce neuropathy, and assessed pain-related endpoints in awake animals on postoperative day 8. The same experimental model was used to assess in vivo electrophysiologic responses of dorsal horn neurons in SNL rats. In the behavioral assessments, palonosetron produced a dose-related reduction in paw withdrawal frequency in SNL rats. In the electrophysiologic studies, palonosetron inhibited spinal neuronal responses evoked by von Frey filaments and spinal response induced by high-temperature stimulation. In the visceral pain studies, palonosetron administered alone inhibited colonic hypersensitivity to mechanical luminal distension in our in vivo rodent model. To further support the proposed goal of our study, we used netupitant, a potent and selective NK₁ antagonist currently under clinical evaluation to treat chemotherapy-induced nausea and vomiting. Other compounds, such as TAK-637, have been evaluated in a variety of experimental models in vitro and in vivo (Venkova et al., 2002; Venkova and Greenwood-Van Meerveld, 2004; Hoffmann-Emery et al., 2006). For netupitant, the affinity for NK₁ receptors was found with a pKᵦ of 8.87 in Chinese hamster ovary cells, whereas in vivo the compound inhibited SP-induced scratching, biting, and licking with a median effective dose of 0.5 mg/kg p.o. (Ness and Gebhart, 1988; Rizzi et al., 2012). In this investigation, netupitant produced a trend toward inhibition of paw withdrawal, although the postinjection values were not significantly different from baseline values. In the electrophysiologic studies, the highest dose of netupitant significantly reduced spinal neuronal responses to
von Frey filaments and spinal neuronal responses to 40, 24, and 48°C stimulation. In the visceral pain studies, we found that in a model of acute visceral hypersensitivity in freely moving rodents, netupitant administered alone inhibited colonic hypersensitivity to mechanical luminal distension.

Taken together, the results from these experiments provided pivotal data on the dose-response effects of palonosetron and netupitant in the in vivo pain assays, and enabled us to determine the noninhibitory doses of each antagonist in the somatic and visceral pain assays, which would be utilized in subsequent experiments to examine potential synergy. Interestingly, the ineffective doses of the compounds were different in the somatic pain models versus the visceral sensitivity assay. The reason for this is currently unclear, but it may reflect the different routes of compound administration, subcutaneous versus oral gavage, and different contributions of 5-HT3 and NK1 receptors to the different pain states. Another possible reason is that the behavioral assays were performed in freely moving rats, whereas the electrophysiologic experiments were performed in anesthetized animals, although the close parallels between the activity of spinal neurons under these conditions and the human pain perception were recently reported (Sikandar et al., 2013). The most likely explanation is the difference between the stimuli; behavior studies have set thresholds, whereas neuronal responses allow pharmacological systems to contribute to suprathreshold responses, which may require higher doses for modulation.

After the experiments to determine the dose-response effects of either palonosetron or netupitant in vivo using a rodent model of somatic and visceral pain, we carried out a series of further experiments. These were designed to investigate whether a combination of a low dose of each compound, which had no effect in the in vivo assays alone, would affect behavioral and electrophysiologic measures of nociception in rats. This study illustrates for the first time that coadministration of palonosetron and netupitant resulted in robust inhibition of behavioral and electrophysiologic indices of somatic and visceral pain. A fundamental question raised by our findings is, by what mechanism does the coadministration of palonosetron and netupitant inhibit somatic and visceral pain when administered at doses shown to be individually ineffective? Although palonosetron does not bind directly with the NK1 receptors, in support of the observations in this study, palonosetron was shown to inhibit SP-induced responses in vitro and in vivo (Stacher, 2002). Specifically, in vitro studies utilizing NG108-15 cells found that preincubation with palonosetron, but not ondansetron and granisetron (other selective 5-HT3 receptor antagonists), inhibited SP-induced calcium mobilization (Plourde et al., 1997; Stathis et al., 2012). In parallel studies in vivo, 10 hours after cisplatin administration and either palonosetron, ondansetron, or granisetron, single neuronal recordings from nodose ganglia were collected after stimulation with SP in a rat model (Rojas et al., 2010b). Palonosetron, but not ondansetron or granisetron, dose dependently inhibited cisplatin-enhanced SP response. Taken together, these results indicate that palonosetron is able to inhibit 5-HT3/NK1 cross-talk both in vitro and in vivo. Results from a previous study, which utilized radioligand binding and functional techniques, demonstrated that palonosetron appears to be an allosteric modulator at the 5-HT3 receptor. This may explain the uniqueness of this compound compared with other orthosteric 5-HT3 antagonists (Rojas et al., 2008) and suggests that palonosetron is a novel pharmacological tool to study in vivo the potential interaction with SP in experimental models of somatic and visceral pain.

Further to the synergism observed by the two compounds in inhibiting 5-HT3/NK1 responses, recent mechanistic studies using NG108-15 cells have shown that palonosetron and netupitant exhibited synergistic effects (Thomas et al., 2014). In these studies, both netupitant and palonosetron induced NK1 receptor internalization in NG108-15 cells, and the receptor internalization was additive when the drugs were used together. In the case of palonosetron, NK1 receptor internalization was 5-HT3 receptor dependent. Furthermore, palonosetron and netupitant independently triggered an increase in protein kinase C (PKC) activity. By contrast, PKC activation was not observed with ondansetron, a structurally different 5-HT3 antagonist. In addition, ondansetron did not have an effect on PKC activation by netupitant. Results suggest enhanced NK1 receptor internalization and PKC activation responses when palonosetron is used in combination with netupitant.

In summary, this study examined whether the two drugs specifically blocking the 5-HT3 and the NK1 receptors could interact in vivo and result in a synergism of efficacy. To address this experimental goal, the behavioral and electrophysiologic responses of two compounds, one a selective 5-HT3 receptor antagonist and the other a selective NK1 receptor antagonist, were studied in vivo using models of somatic and visceral pain. We discovered that a combination of palonosetron, a 5-HT3 receptor antagonist, with netupitant, a NK1 receptor antagonist, showed synergistic analgesic activity, and this combination approach may prove to be a useful therapeutic approach to treat pain associated with IBS and/or other gut disturbances.

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Authorship Contributions
Participated in research design: Greenwood-Van Meerveld, Pietra, Dickenson. Conducted experiments: Mohammadi, Tyler. Contributed new reagents or analytic tools: Greenwood-Van Meerveld, Pietra. Performed data analysis: Mohammadi, Tyler. Wrote or contributed to the writing of the manuscript: Greenwood-Van Meerveld, Mohammadi, Tyler, Pietra, Dickenson.

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