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

Beverley Greenwood-Van Meerveld, Ehsan Mohammadi, Karl Tyler, Claudio Pietra, Lucy A. Bee, and Anthony Dickenson

Department of Physiology (B.G.-V.M.), Veterans Affairs Medical Center (B.G.-V.M.), Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center (B.G.-V.M., E.M., K.T.), Oklahoma City, Oklahoma; Research and Preclinical Department, Helsinn Healthcare SA, Lugano, Switzerland (C.P.); and Department of Neuroscience, Physiology, and Pharmacology, University College London, London, United Kingdom (L.A.B., A.D.)

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ABSTRACT

Synergistic activity has been observed between serotonergic 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 antinociception 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.
stimulation (Hayashida et al., 2012). In patients with IBS associated with diarrhea, 5-HT₃ receptor antagonism was shown to reduce symptoms (Whitehead et al., 1990; Coates et al., 2006), but the unfavorable side-effect profile of alosetron, a selective 5-HT₃ receptor antagonist, has limited the clinical use of this approach.

Another neurotransmitter released from the central terminals of primary afferent fibers entering the spinal cord and implicated in chronic pain responses is the tachykinin substance P (SP) with neurokinin 1 (NK₁) receptors serving as the postsynaptic targets for SP (Gaudreau and Plourde, 2003; Wang et al., 2011). However, only about 15% of afferent release SP, and this may be further reduced after nerve injury, although some studies report a phenotypic switch of the transmitter production and release to larger fibers. NK₁-expressing neurons in the superficial dorsal horn are critical to spinal hypersensitivity, and drive both spinal wind-up and the ascending projection that eventually trigger the descending facilitatory neurons in the superficial dorsal horn are critical to spinal hyperactivity (Wang et al., 2011). Studies in a guinea pig model demonstrated that central NK₁ receptors play a significant role in colonic hypersensitivity induced by visceral afferent nerve sensitization from gastrointestinal origin or acute psychosomatic stress, but not in the perception of colorectal distension in animals with normosensitive colon (Greenwood-Van Meerveld et al., 2003). Experimental evidence has described the effect of compounds endowed with 5-HT₃ or NK₁ antagonist activity in inducing analgesic response in nerve injury (Suzuki et al., 2002, 2003, 2004) or in visceral inflammatory pain models (Chang, 2005; Parenti et al., 2012). More recently, synergistic activity was observed between serotonergic 5-HT₃ and tachykinergic NK₁ responses in vitro and in vivo (Rojas et al., 2010a; Stathis et al., 2012). In support of this, there is experimental evidence for cross-talk between spinal 5-HT₃ and NK₁ receptor–mediated mechanisms in modulating biologic responses. For example, SP, a NK₁ receptor agonist, was shown to potentiate 5-HT₃ receptor–mediated inward current in rat tririgeminal ganglion neurons (Hu et al., 2004). In addition, NK₁ antagonism blocked 5-HT–induced vagal afferent activation (Minami et al., 2001). In this study, we examined whether 5-HT₃ 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 ligation 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 (130–150 g at time of spinal nerve ligation (SNL) surgery) were obtained from Central Biologic Services (University College London, London, UK). Rats were housed in the Biologic Services Unit in standard cages under a 12-hour light/dark cycle with ad libitum access to food and water. All animal experiments were approved by the UK Home Office and were carried out in accordance with guidelines set by personal and project licenses, and thus complied with the UK Animals (Scientific Procedures) Act 1986. All efforts were made to minimize animal suffering and to reduce the number of animals used.

SNL Surgery. An aseptic technique was used for all surgical procedures. Experimental neuropathic pain was established by tightly ligating the L5 and L6 spinal nerves as previously described (Kim and Chung, 1992). Rats were anesthetized (1:1 O₂/N₂O, 3% halothane for induction, 1% maintenance). A small left-side incision was made at approximately L4-S2, and paraspinal muscle and fat were removed from spinous processes. Part of the L6 transverse process was clipped with rongeurs to expose the parallel-lying L4 and L5 spinal nerves. The L5 nerve was isolated and hooked with a finely pulled glass rod and tightly tied with nonabsorbable 6-0 silk thread distal to the dorsal root ganglion (and proximal to the formation of the sciatic nerve). The L6 nerve was then hooked from under the sacrum and tied in a similar way. Hemostasis was confirmed and the wound was sutured with 3-0 absorbable silk. The surrounding skin was pulled together and secured over the incision with wound clips. Rats recovered in an incubator, and upon confirmation that they had no observable motor impairment in their left hindpaw, were returned to home cages.

Somatic Sensitivity Assessment. On postoperative day 8, behavioral signs of punctate mechanical hypersensitivity were assessed in the left ipsilateral hindpaw in awake and alert rats. Rats were individually placed in clear acrylic cubes on an elevated floor of wire mesh, and after a period of acclimation (30 minutes), mechanical sensitivities were determined by paw withdrawal to von Frey filaments with a bending force of 6 g. The filament was applied to the plantar surface of the paw for approximately 2 to 3 seconds with enough force to cause buckling; for each animal, this was repeated 10 times at set positions on each paw. The number of lifts in response to the filament was noted for each rat. Withdrawal frequency was quantified as the number of foot withdrawals per 10 trials.

Electrophysiology. Electrophysiology experiments were conducted on postoperative days 14–17 in anesthetized rats. The evoked responses of dorsal horn lamina V–VI neurons to stimulation of the peripheral receptive field were recorded. Mechanical (von Frey filaments of 2g, 5g, 26g, and 60g applied for 10 seconds) and thermal (40, 45, and 48°C applied for 10 seconds) stimuli were assessed. After three stable baseline responses, palonosetron or netupitant was administered by s.c. injection. Mechanical and thermal tests were carried out at 10, 30, and 50 minutes after each dose.

Experimental Series 2: Assessment of Visceral Pain Behavioral Responses

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 experimentation. 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 1 week. 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
than 0.05.

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 adapter onto the abdominal oblique muscle and was connected via an electroencephalogram (EEG) preamp (Marantz, Mahwah, NJ). The EEG was connected to a Model 7 Polygraph (Grass Technologies, Warwick, RI). The EEG was set at 0.02 mV/cm sensitivity and was connected to a Modexia 7A polygraph (Modexia, La Jolla, CA). For all data, a 95% confidence interval was used as a measure of statistical significance. In experimental series 1, statistical significance with respect to behavioral scores was calculated using nonparametric Wilcoxon matched-pairs tests. For electrophysiological data, statistical analyses were performed on raw data using two-way analysis of variance for responses to mechanical and thermal stimuli, and if significant, Bonferroni post hoc tests were performed. For responses to electrical stimulation, a one-way analysis of variance was used followed by Dunnett’s post hoc multiple comparisons test for significant values. In experimental series 2, to determine statistical significance between multiple control and treatment groups, data were compared using one-way analysis of variance followed by a Bonferroni post test. Results were deemed significant when P values were less than 0.05.

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, electrophysiological 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 pKb 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, a selective 5-HT3 receptor antagonist, 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 cispilatin-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 synergy 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 using in vivo 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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Address correspondence to: Dr. Claudio Pietra, Research and Preclinical Department, Heclinn Healthcare SA, 6915 Lugano, Switzerland. E-mail: claudio.pietra@helsinn.com