The antiemetic 5-HT₃ receptor antagonist palonosetron inhibits Substance P-mediated responses in vitro and in vivo

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Running Title Page

Running title: Palonosetron inhibits SP-mediated responses

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ABBREVIATIONS: CINV, chemotherapy induced nausea and vomiting; MEC, moderately emetogenic chemotherapy; 5-HT, serotonin; SP, substance P.

Recommended Section Assignment: Cellular and Molecular
Abstract

Palonosetron is the only 5-HT₃ receptor antagonist approved for the treatment of delayed chemotherapy-induced nausea and vomiting (CINV) in moderately emetogenic chemotherapy (MEC). Accumulating evidence suggests that Substance P (SP), the endogenous ligand acting preferentially on NK-1 receptors, not serotonin, is the dominant mediator of delayed emesis. However, palonosetron does not bind to the NK-1 receptor. Recent data has revealed crosstalk between the NK-1 and 5HT₃ receptor signaling pathways; we postulated that if palonosetron differentially inhibited NK-1/5-HT₃ crosstalk, it could help explain its efficacy profile in delayed emesis. Consequently, we evaluated the effect of palonosetron, granisetron and ondansetron on SP-induced responses in vitro and in-vivo. NG108-15 cells were preincubated with palonosetron, granisetron or ondansetron; antagonists were removed and the effect on serotonin enhancement of SP-induced calcium release was measured. In the absence of antagonist, serotonin enhanced SP-induced calcium-ion release; following preincubation with palonosetron, but not ondansetron or granisetron, inhibited the serotonin enhancement of the SP response. Rats were treated with cisplatin and either palonosetron, granisetron or ondansetron. At various times post-dosing, single neuronal recordings from nodose ganglia were collected following stimulation with SP; nodose ganglia neuronal responses to SP were enhanced when the animals were pretreated with cisplatin. Palonosetron, but not ondansetron or granisetron, dose-dependently inhibited the cisplatin-induced SP enhancement. The results are consistent with previous data showing that palonosetron exhibits distinct pharmacology versus the older 5-HT₃ receptor antagonists and provide a rationale for the efficacy observed with palonosetron in delayed CINV in the clinic.
Introduction

Nausea and vomiting are common, severe and feared side effects of many chemotherapeutics. Inadequate control of chemotherapy induced nausea and vomiting (CINV) impairs functional activity and may compromise adherence to treatment. 5-HT3 receptor antagonists are most efficacious against acute emesis (0-24 h after chemotherapy administration) whereas NK-1 receptor antagonists have been associated with the prevention of delayed emesis (24 – 120 h after chemotherapy administration) (Hesketh et al., 2003). Even though the terms acute and delayed are approximations with no clear distinction of when acute emesis ends and delayed emesis begins, they point to the idea that different mechanisms are at play. Palonosetron is unique among 5-HT3 receptor antagonists in that in addition to being effective against acute emesis, it has shown efficacy against delayed emesis (Eisenberg et al., 2003; Aapro et al., 2006; Saito et al., 2009). The reason for this unique efficacy in delayed emesis is not clear since palonosetron does not bind to NK-1 receptors (Wong et al., 1995).

Receptor crosstalk, defined as activation of one receptor by its ligand affecting cellular responses to another receptor system(s), has been well described. Recent reports in the literature have shown that there is crosstalk between NK-1 and 5-HT3 receptor signaling pathways. For example, substance P (SP), an agonist at the NK-1 receptor, was shown to potentiate 5-HT3 receptor mediated inward current in rat trigeminal ganglion neurons (Hu et al., 2004). In separate studies, 5-HT3 receptor antagonists were shown to block SP—mediated vagal afferent activation (Minami et al., 2001). In addition, NK-1 antagonism blocked serotonin-induced vagal afferent activation (Minami et al., 2001). Evidence of receptor signaling crosstalk raises the interesting possibility that palonosetron’s unique efficacy in delayed emesis could be due to differential inhibition of the 5-HT3/NK-1 receptor crosstalk. In the present work, we evaluated the effect of
palonosetron, granisetron and ondansetron on SP-induced responses in vitro and in-vivo. We report that palonosetron inhibited the serotonin enhancement of the SP response in NG108-15 cells and the cisplatin enhancement of the neuronal response to SP in rat nodose ganglia. This inhibition was not observed with ondansetron or granisetron, two other widely used 5-HT3 receptor antagonists. The results suggest palonosetron differentially inhibits 5-HT3/NK-1 receptor signaling crosstalk and provide a rationale to explain palonosetron’s unique efficacy against delayed emesis in the clinic.
Methods

Calcium-ion release measurements in NG108-15 cells

NG108-15 cells, known to express both 5-HT$_3$ and NK-1 receptors, were grown in high glucose DMEM supplemented with a mixture of sodium hypoxanthine, aminopterin and thymidine (HAT) and 10% heat inactivated fetal bovine serum and 2 mM glutamine to 90% confluency. Cells were incubated with palonosetron (10 nM), granisetron (60 nM) and ondansetron (300 nM) for 2 h. Antagonist concentrations were approximately 50-fold $K_d$ in each case to make sure receptors were saturated, based upon $K_d$ values previously described in NG108-15 or N1E-115 cells (Wong et al., 1995; Rojas et al., 2008). Subsequently, antagonists were removed and cells were incubated with growth media without antagonist for one additional hour to allow for dissociation of antagonists still bound to the cell. Next, cell media was replaced with isosmotic HEPES buffer (20 mM, pH 7.4, 130 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$ and 2 mM CaCl$_2$) containing 2 µM Fluo-4 acetoxymethyl (AM) ester and pluronic acid (0.04%). Pluronic acid was added as nonionic surfactant to sequester the AM ester molecules into micelles for cell uptake. Cells were incubated for 1 h to allow for cell uptake of the AM ester. Cells were then incubated with SP at various concentrations for 1 h in HEPES buffer without the AM ester and pluronic acid. Subsequently, measurement of calcium-ion release due to SP alone or to SP plus serotonin (10$^{-7}$ M final concentration added immediately before measurement) was made using the FLIPR™.

Animal Preparation

Studies were conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and use of Laboratory Animals as adopted and promulgated by the National Institutes
of Health. Experiments were performed on adult male Sprague Dawley rats weighing 270 to 350 g. Animals were housed in an animal facility with limited access. Room temperature was 22 ± 2 °C and relative humidity was set at 55 ±10%. Artificial lighting was provided 24 h with a cycle of 12 h light / 12 h dark (light 7 a.m. – 7 p.m.). Animals had free access to food and water; they were anesthetized with an i.p. injection of a mixture of α-chloralose (80 mg/kg) and urethane (800 mg/kg). The anesthetic was supplemented every 3.5 h with an i.v. dose of ¼ of the initial dose. Adequate depth of anesthesia was established in prior experiments by monitoring heart rate and withdrawal reflexes after subcutaneous electrical stimulation or pinching of the skin.

Animals were ventilated with a respirator; a tracheal tube permitted artificial ventilation with room air (75 – 85 strokes/min, 3.5-4.0 cm³ tidal volumes). A midline abdominal incision exposed the abdominal vagus, the stomach, and the duodenum. Stimulation of the subdiaphragmatic vagus nerve was accomplished by placing a pair of Teflon-coated, pure gold wire electrodes (outside diameter, 76 µm) around the anterior and posterior trunks, about 2 to 3 cm above the gastroesophageal junction and above the accessory and celiac branches of the vagus nerve. These electrodes were loosely sutured to the esophagus to limit displacement.

**Drug Dosing**

Cisplatin (5 mg/kg) was given i.p., SP (10 µg/kg) was given by intra-arterial injection and antagonists were administered by i.v. infusion. Antagonists were dissolved in 0.9 % saline solutions and infused at a rate of 0.085 ml/min. Total dose of 38 mg/kg for ondansetron was administered over 30 min in 360 µg/100 µL solution. Total dose of 840 µg/kg for granisetron was administered over 10 min in 24 µg/100 µL solution. Total doses of 30, 100 and 300 µg/kg for palonosetron were administered over 10 min in 0.9, 3 and 9 µg/100 µL solutions respectively.
Recording of single nodose neuronal activity

Rats were placed in a small Kopf animal stereotaxic frame. Body temperature was maintained with a heating pad. The right nodose ganglion was exposed by a short dorsal approach. Using an operating microscope, the ganglion sheath was removed and separated from the adjacent cervical sympathetic trunk and carotid artery. The recording microelectrodes were pulled from glass capillaries (A-M Systems, Everett, WA) using a micropipette puller and microelectrode beveler to obtain tips ranging between 0.08 to 0.1 µm in diameter with a resistance of 50 to 70 MΩ. The beveled glass micropipette filled with 1.0 M KCl was lowered into the nodose ganglion. Once a nodose ganglion neuron activated by the electrical vagal stimulation was identified, the response of that neuron to intra-arterial injection of SP was measured. Only gastrointestinal C-fibers were recorded. The basal discharge was monitored for 2 min to confirm the stability of the basal firing frequency. Results shown in Fig. 2 – Fig. 5 are the average from 12 -42 measurements made on isolated neurons obtained from 7-26 rats.

Statistical Analysis

Graph Pad PRISM® was used to obtain EC₅₀ values in in vitro experiments (sigmoidal dose response variable slope). Errors correspond to S.E.M. of at least 8 independent determinations. Student’s t-test (two-tailed distribution) was used to determine p values. Experimental groups in the in vivo experiments included at least 12 independent neuronal measurements from at least 7 rats to obtain average values. Error bars correspond to S.E.M.
Results

Palonosetron, but not ondansetron or granisetron, inhibited serotonin enhancement of SP-induced calcium-ion release in NG-108-15 cells. Calcium-ion release in NG-108-15 cells was dependent on SP concentration with an EC$_{50}$ of 6.7 ± 0.97 µM; when serotonin (10$^{-7}$ M) was present, the EC$_{50}$ shifted 10-fold to the left to 0.62 ± 0.09 µM (Fig. 1A, Table 1). Serotonin alone did not have an effect on internal calcium release or calcium influx at 10$^{-7}$ M (data not shown). Serotonin enhancement of the SP response was not affected by prior incubation with ondansetron or granisetron; EC$_{50}$ values were the same to those obtained with SP plus serotonin (Fig. 1B, 1C and Table 1). In contrast, preincubation with palonosetron inhibited the serotonin enhancement of the SP response; the EC$_{50}$ shifted 6-fold to the right to 3.7 ± 0.84 µM. ***p < 0.001 compared to the EC$_{50}$ for SP and 5-HT (Fig. 1D, Table 1).

Cisplatin enhanced the neuronal response to SP in nodose ganglia. The resting discharge of single nodose neurons following intra arterial administration of vehicle was negligible (< 1 impulse/10s). When SP was administered at 10 µg/kg via intra-arterial injection the neuronal activity was increased, although variable, to 4 ± 4 impulses/10 s. When measurement of the SP response was made 10 h after i.p. administration of 5 mg/kg i.p. cisplatin (Fig. 2A), SP responses increased to 23 ± 3 impulses/10 s. When the dose of cisplatin was increased to 10 mg/kg cisplatin, both, basal and SP responses were similar within experimental error to when 5 mg/kg cisplatin was used (Fig. 2B). In short, there was approximately 6-fold increase in the neuronal response to SP in the presence of 5-10 mg/kg cisplatin.

Palonosetron dose-dependently inhibited cisplatin’s potentiation of the SP response in nodose ganglia. Preliminary studies with the three antagonists suggested that only palonosetron
had an effect on SP responses. Consequently, we conducted a dose response with palonosetron. Cisplatin (5 mg /kg i.p.) was administered to rats; ten hours later, single nodose ganglia neurons were isolated and four electrophysiological recordings were made at 10 minute intervals (Fig. 3A). First, a basal reading was taken. Second, the neuronal response to SP (10 µg/kg intra-arterial injection) was measured. Third, the neuronal response to palonosetron (i.v. infusion of 30, 100 and 300 µg/kg) was measured. The fourth and final recording measured neuronal responses after the administration of SP (10 µg/kg intra-arterial injection). The first three responses were similar throughout the experiment: basal readings were 7.5 ± 3 impulses/10 sec SP increased the neuronal activity to 38 ± 4 impulses/10 sec, and palonosetron alone at 30, 100 or 300 µg/kg did not have an effect on either basal or SP-induced responses. However, when SP was administered following palonosetron, the neuronal responses dose dependently decreased to 32 ± 4 (SP + 30 µg/kg palonosetron), 18 ± 2 (SP + 100 µg/kg palonosetron) and 9 ± 3 impulses/10 sec (SP + 300 µg/kg palonosetron) (Fig. 3B).

**Ondansetron and granisetron had no effect on the cisplatin enhancement of the SP response in nodose ganglia.** Since palonosetron exhibited inhibition of the cisplatin-induced SP response, we explored more thoroughly whether the effect was common to other 5-HT3 receptor antagonists such as granisetron and ondansetron. In an attempt to replicate the results with ondansetron and granisetron, experimental doses which accounted for their differences in clinical dose vs. palonosetron were chosen. Specifically, we calculated the experimental dose by taking the difference in clinical dose between palonosetron and ondansetron (approximately 128-fold) and multiplying it by the most effective palonosetron dose tested in our model (300 µg/kg x 128 = 38 mg/kg). Similarly, the dose for granisetron was calculated by the difference in clinical dose between palonosetron and granisetron (approximately 2.8-fold) multiplied by the most effective
palonosetron dose (300 µg/kg x 2.8 = 840 µg/kg). The doses employed for ondansetron and granisetron in these studies turned out to be in excess of their active doses reported in previous efficacy studies in animals (Eglen et al., 1995; Rudd and Naylor, 1996; Endo et al., 1999; Rudd et al., 2002). Vagal nerve activity was measured in animals 10 h after cisplatin administration (5 mg /kg i.p.). The same measurements as in the palonosetron dose dependence experiment were made: basal, response to SP, response to each antagonist alone, and response to administration of SP (Fig. 4A). Basal and SP responses were 8 ± 3 and 33 ± 6 impulses/10 s, respectively.

Neither ondansetron (38 mg/kg i.v.) nor granisetron (840 µg/kg i.v.) had an effect on the basal response. When SP was administered following ondansetron or granisetron, the SP response was not affected. This lack of effect was in stark contrast to over 70% inhibition of the SP response following palonosetron administration (Fig. 4B).

**Palonosetron’s inhibition of the cisplatin-induced SP-response was time dependent.** One major consideration in these experiments as well as in the clinic is the time of administration of 5-HT₃ receptor antagonist with respect to the time of administration of the chemotherapeutic agent. In the experiments described thus far, the antagonists were administered 10 h after cisplatin. We wanted to explore whether inhibition of the SP response could be observed at earlier time points. To this end, we explored the inhibition of the neuronal response to SP when antagonists were administered 30 min before and 5 h after cisplatin. As above, the measurement of the neuronal response to SP was made 10 h after cisplatin (Fig. 5A). The neuronal response to SP administration in the presence of cisplatin was 32 ± 4 impulses/10 s. When ondansetron (38 mg/kg i.v.) or granisetron (840 µg/kg i.v.) were given at either 30 min before or 5 and 10 h after cisplatin, there was no effect on the SP response. In contrast, when palonosetron (300 µg/kg i.v.) was administered at 30 min before or 5 and 10 h after cisplatin, inhibition of the SP response was
observed at every time point. There was $33 \pm 7\%$ inhibition when palonosetron was administered 30 min before cisplatin, $70 \pm 8\%$ inhibition when palonosetron was given 5 h after cisplatin and $78 \pm 10\%$ inhibition when palonosetron was given 10 h after cisplatin respectively (Fig. 5B).
Discussion

5-HT3 and NK-1 receptor antagonists are common therapy to help prevent CINV. In general, 5-HT3 receptor antagonists are efficacious in the acute (0-24 h) phase of CINV whereas NK-1 receptor antagonists are most efficacious during the delayed (24–120 h) phase (Hesketh et al., 2003). Palonosetron is the only 5-HT3 receptor antagonist that is labeled for the treatment of delayed emesis in MEC. The mechanisms by which palonosetron helps prevent delayed emesis are not known; this is tantalizing given that palonosetron does not bind to the NK-1 receptor (Wong et al., 1995). On the other hand, palonosetron has been shown to exhibit unique molecular interactions with the 5-HT3 receptor. Palonosetron exhibits allosteric binding and positive cooperativity (Rojas et al., 2008) and triggers receptor internalization as well as long-term inhibition of receptor function (Rojas et al., 2010), attributes not shared by other 5HT3 receptor antagonists.

Various reports in the literature have shown that there is crosstalk between NK-1 and 5-HT3 receptor signaling pathways (Hu et al., 2004). SP, a known agonist at the NK-1 receptor, was shown to potentiate 5-HT3 receptor mediated inward current in rat trigeminal ganglion neurons. Potentiation of 5-HT3 receptor current through SP is thought to involve second messenger signaling that culminates in PKC activation (Hu et al., 2004). In addition, 5-HT3 receptor antagonists have been shown to block SP-mediated vagal afferent activation and NK-1 antagonists were shown to block serotonin-induced vagal afferent activation (Minami et al., 2001). Evidence of receptor signaling interaction raises the interesting possibility that palonosetron’s unique efficacy in delayed emesis could be due to differential inhibition of the 5-HT3/NK-1 receptor crosstalk. In the present work, we evaluated the effect of palonosetron, granisetron and ondansetron on SP-induced responses in vitro and in-vivo.
NG108-15 cells were used in the in vitro studies because they are known to express both the 5-HT3 and NK-1 receptors (Reiser and Hamprecht, 1989; Emerit et al., 1993). Previous studies have also shown that SP, acting through the NK-1 receptor, stimulates an increase of intracellular calcium ions through a release of intracellular calcium-ion stores in Chinese hamster ovary cells (Garland et al., 1996). Consequently, we determined the dependence of intracellular calcium ion release on SP concentration and the potential synergistic response in the presence of serotonin in NG108-15 cells. Intracellular calcium-ion release was dependent on SP concentration (EC50 = 6.7 ± 0.97 µM). In addition, serotonin, at a subthreshold concentration (10⁻⁷M), where it did not elicit calcium-ion mobilization by itself, induced a 10-fold potency increase of the response to SP alone (EC50 = 0.62 ± 0.09 µM, Fig. 1A, Table 1). This result was in agreement with previous findings pointing to interactions between the signaling of 5-HT3 and NK-1 receptors in rat trigeminal ganglion neurons, where serotonin potentiation of inward current was enhanced by SP pre-application (Hu et al., 2004).

Further, NG108-15 cells provided a simple in vitro system to evaluate the potential differential inhibition of 5-HT3/NK1 receptor crosstalk by 5-HT3 receptor antagonists. Recently we have shown that palonosetron triggers long term 5-HT3 receptor internalization whereas ondansetron and granisetron exhibit simple receptor blockade (Rojas et al., 2010). The purpose of these in vitro experiments was to determine if pre-incubation with the 5-HT3 antagonists, followed by their complete removal from the media, could have a persistent downstream effect on SP function. In other words, could 5-HT3 receptor internalization effect serotonin’s activation of SP-induced calcium-ion release? To ensure excess drug availability for receptor saturation, we chose to use antagonists concentrations >15-fold their respective IC50 values needed to inhibit serotonin-induced calcium-ion influx (Supplemental Methods and Supplemental Figures 1A – C;
IC₅₀s were 0.7, 2 and 3 nM for palonosetron, granisetron and ondansetron respectively. These concentrations were also approximately 50 times their previously determined K_d values (Wong et al., 1995; Rojas et al., 2010). When cells were preincubated with excess palonosetron (10 nM) followed by the drug’s removal through extensive cell washing, the serotonin effect on the response to SP was inhibited 6-fold (rightward shift of the EC₅₀ curve, Fig.1B, Table 1). In contrast, when cells were preincubated with excess ondansetron (300 nM) or granisetron (50 nM) followed by their removal, there was no inhibition of the serotonin-induced SP response (Fig. 1C and 1D and Table 1). Cell washing to remove the antagonists from the media took into account their respective half-lives of dissociation from 5-HT₃ receptors to insure that all antagonist bound to the cell surface was dissociated before calcium-ion flux measurements were initiated (Rojas et al., 2008). The results showed that exposure to palonosetron uniquely inhibited the serotonin-induced activation of the SP response in vitro even after the drug was removed from the media.

These in vitro results are likely due to palonosetron’s distinctive ability to induce 5-HT₃ receptor internalization and cause long term reduction in 5-HT₃ receptor density on the cell surface.

Previously we have shown palonosetron rapidly partitions inside cells along with the 5-HT₃ receptor during antagonist pre-incubation; once inside the cell, palonosetron does not re-appear in the extracellular milieu for at least 2 hours. Importantly, palonosetron’s internalization is dependent on the presence of the 5-HT₃ receptor, as palonosetron was shown not to partition into cells that do not express the 5-HT₃ receptor (Rojas et al., 2010). These data suggests that palonosetron’s unique effect on inhibition of serotonin-induced SP activation is a 5-HT₃ receptor-mediated difference, not a cell permeability difference.

In order to determine if the in vitro results could also be demonstrated in vivo, we examined neuronal responses in nodose ganglia to SP after cisplatin administration in rats. We used rat
nodose ganglia because they have been shown to express both 5-HT₃ and NK-1 receptors (Hu et al., 2004); and cisplatin because it is known to activate the mechanisms of both acute and delayed emesis (Hesketh, 2008). Cisplatin triggers the release of serotonin from enterochromafin cells which in turn activates 5-HT₃ receptors located on the surface of vagal afferents. Consistent with previous data showing that 5-HT₃ receptor activity can influence NK-1 signaling (Minami et al., 2001; Hu et al., 2004), we found that pretreatment of rats with cisplatin induced a 3 to 6-fold increase of the neuronal response in nodose ganglia to SP (Fig. 2) in agreement with a previous report (Wu et al., 2009).

Since cisplatin potentiated the SP response in nodose ganglia, we explored the possibility that 5-HT₃ receptor antagonists could inhibit the cisplatin-induced activation of the SP response. We found that palonosetron uniquely inhibited the cisplatin-induced neuronal response to SP in a dose dependent manner 10 h after cisplatin administration (Fig. 3 and 4). Neither ondansetron, nor granisetron had an effect. Importantly, the doses of ondansetron and granisetron in these studies took into account differences in clinical dose and were higher than those used in animal models where efficacy with these antagonists was observed (Eglen et al., 1995; Rudd and Naylor, 1996; Endo et al., 1999; Rudd et al., 2002). Consequently, the lack of inhibition of SP response with the high doses of granisetron and ondansetron employed, obviated the need for evaluating the efficacy of lower doses. One limitation, however, is that even though these antagonists are thought to be selective for the 5-HT₃ receptor, the compounds could be acting on off target sites that could affect neuronal activity in the nodose ganglia at the relatively high doses used. Minami and coworkers reported that granisetron inhibits the SP response under acute conditions in vivo (Minami et al., 2001). However our in vivo study was different in that it was designed to explore the effect of 5-HT₃ receptor antagonists on the delayed cisplatin-induced
plasticity changes characterized by an enhanced SP response (Wu et al., 2009).

Correspondingly, the experiment outlined in Fig. 4 involved measurements of the SP response 10 h after cisplatin administration and the potential effects of 5-HT3 receptor antagonists on this enhancement. Under these conditions only palonosetron inhibited the enhanced interaction of SP and serotonin in vagal afferent neurons. These differences are likely due to palonosetron-triggered 5HT3 receptor internalization that is unique and would be predicted to provide more persistent 5HT3 receptor functional inhibition compared to simple binding followed by dissociation at the receptor on the cell surface as occurs with ondansetron and granisetron.

Since the time of antiemetic administration could have different effects on cisplatin-induced toxicity in the clinic, we also explored the time dependence of palonosetron inhibition of the cisplatin-induced neuronal response to SP and the potential that ondansetron and/or granisetron could also have an effect if administered at times other than 10 h after cisplatin administration.

Additional studies were performed where antagonists were given half-hour before cisplatin and 5 h after cisplatin. When ondansetron or granisetron were used, no inhibition of the cisplatin effect was observed at either time point. In contrast, when palonosetron was administered half-hour before cisplatin or 5 hours after cisplatin and the effect on SP activity was measured 10 h later, 33 ± 7% and 70 ± 8% inhibition was observed, respectively (Fig. 5). In short, palonosetron inhibition of the SP response could be seen when palonosetron was administered 10 h, 5 h or 10 min before measurement of the SP response.

The half-life of palonosetron in the rat is 90 min after i.v. administration (Supplemental Palonosetron Rat PK) which is in the same order of magnitude as the half-lives previously reported for ondansetron (20 – 40 min) (Yang and Lee, 2008) and granisetron (50 min) (Huang
et al., 1999) in rats. Therefore the differences we observed are likely not due to differences in pharmacokinetics.

In summary, palonosetron uniquely inhibits crosstalk between the 5-HT$_3$ and NK-1 receptor pathways in a dose-dependent and time dependent fashion. Our results are consistent with previous data showing that palonosetron exhibits distinct pharmacology versus the older 5-HT$_3$ receptor antagonists and provide a rationale for the unique efficacy observed with palonosetron in delayed CINV in the clinic.
References


Footnotes

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Research work was performed while CR, JZ, MS, JA, AGT and BSS were employees at Eisai, Inc. and while YL was at the University of Michigan, Division of Gastroenterology, Ann Arbor, Michigan, USA

CR and YL contributed equally to this work
Legends for figures

Fig. 1. The effect of 5-HT₃ receptor antagonists on serotonin activation of SP–induced intracellular calcium-ion release. (A) Serotonin activation of the SP response. NG108-15 cells were incubated with SP at various concentrations and after one hour, internal calcium release was measured upon the addition of ± serotonin (10⁻⁷ M). (B) Ondansetron (30 nM) was pre-incubated with cells for 2 h; antagonist was subsequently removed and the effect on serotonin activation of the SP response was measured. Responses to SP and to SP + 5HT in (A) are shown for reference. (C) And (D) same as (B) except granisetron (5 nM) and palonosetron (1 nM) were used respectively. Error bars correspond to ± SEM; error bars in (B), (C) and (D) for the SP and SP + 5HT traces are the same as in (A). Each EC₅₀ curve corresponds to the average of at least 8 independent determinations.

Fig. 2. The effect of cisplatin on neuronal response to SP stimulation. (A) Experimental protocol. Cisplatin was given at 0 h; 10 h later, basal and SP responses were measured. (B) Animals were anesthetized after cisplatin administration, vagal nerve was isolated and single neuronal responses were examined. Prior to SP stimulation basal discharge frequency (impulses/10 s) was assessed for 1 min. The discharge frequency after SP administration was subsequently measured for 3 min. Results are the average of at least 12 independent neuronal measurements from at least 7 rats (*** p < 0.001 compared to no cisplatin). Error bars correspond to ± S.E.M.

Fig. 3. The effect of dose of palonosetron on cisplatin-induced neuronal response to SP. (A) Experimental protocol. Cisplatin was given and 10 h later 4 different measurements of neuronal activity were made at 10 min intervals. (1) Basal measurement, (2) response to SP, (3) response to palonosetron alone and (4) response to SP following palonosetron administration. (B)
Neuronal response to SP following palonosetron administration at 30, 100 and 300 µg/kg; only the 4th measurement at each concentration is shown. Results are the average of at least 12 independent neuronal measurements from at least 7 rats (* p < 0.05; *** p < 0.001 compared to vehicle). Error bars correspond to ± S.E.M.

Fig. 4. Effects of 5-HT3 receptor antagonists on the cisplatin-induced neuronal response to SP. (A) Experimental protocol. Cisplatin was given and 10 h later 4 different measurements of neuronal activity were made at 10 min intervals. (1) Basal measurement, (2) response to SP, (3) effect on baseline of ondansetron, granisetron or palonosetron and (4) response to SP following ondansetron, granisetron or palonosetron. (B) Set of four measurements for each antagonist as described in (A). Results are the average of at least 12 independent neuronal measurements from at least 7 rats (*** p < 0.001 compared to SP). Error bars correspond to ± S.E.M.

Fig. 5. Effect of time of administration of 5-HT3 receptor antagonists on the cisplatin-induced neuronal response to SP. (A) Experimental protocol. Ondansetron, granisetron and palonosetron were given 0.5 h before cisplatin and 5 and 10 h after cisplatin. Four separate groups of animals were used: (1) cisplatin control, (2) ondansetron, granisetron or palonosetron administered 0.5 h before cisplatin, (3) 5 h after cisplatin and (4) 10 h after cisplatin. Response to SP was measured 10 h after cisplatin administration. (B) SP response after each antagonist was administered at different times with respect to cisplatin. Results are the average of at least 12 independent neuronal measurements from at least 7 rats (*p < 0.05; *** p < 0.001 compared to cisplatin). Error bars correspond to ± S.E.M.
Tables

**Table 1** – Effect of 5-HT\textsubscript{3} receptor antagonists on serotonin activation of SP response in NG-108-15 cells. EC\textsubscript{50} values correspond to the results shown in Fig. 1. Graph Pad PRISM\textsuperscript{®} was used to obtain EC\textsubscript{50} values (sigmoidal dose response variable slope). Errors correspond to ± S.E.M. Data is the average of at least 8 independent determinations. EC\textsubscript{50} for SP + 5-HT + palonosetron was statistically different with p < 0.001 when compared to SP + 5-HT using student’s t test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC\textsubscript{50} ± SEM (µM)</th>
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<tr>
<td>SP</td>
<td>6.7 ± 0.97</td>
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<tr>
<td>SP + 5-HT</td>
<td>0.62 ± 0.09</td>
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<tr>
<td>SP + 5-HT + ondansetron</td>
<td>0.66 ± 0.17</td>
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<tr>
<td>SP + 5-HT + granisetron</td>
<td>0.57 ± 0.10</td>
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<tr>
<td>SP + 5-HT + palonosetron</td>
<td>3.7 ± 0.84</td>
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Fig. 1 (A)

Ca²⁺ Internal Release
Fluoresc. Counts 12-150 s

SP
SP + 5HT

SP Concentration (log scale)

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Fig. 1 (B)

Ca$^{2+}$ Internal Release
Fluoresc. Counts 12-150 s

Ondansetron

SP
SP + 5HT
SP + 5HT + Ondan

SP concentration
Fig. 1 (C)

The graph shows the relationship between SP concentration and Ca$^{2+}$ internal release fluoresecent counts (12-150 s). The x-axis represents the SP concentration, while the y-axis represents the Ca$^{2+}$ internal release fluoresecent counts. The graph compares the effects of SP, SP + 5HT, SP + 5HT + Granisetron, and Granisetron alone on Ca$^{2+}$ internal release.

**Granisetron**

**SP**

**SP + 5HT**

**SP + 5HT + Granisetron**
Fig. 1 (D)

- Palonosetron
- SP
- SP + 5HT
- SP + 5HT + Palo

Ca\(^{2+}\) Internal Release Fluoresc. Counts 12-150 s

SP Concentration
Fig. 2 (A)

Time: 0 h

Cisplatin or Vehicle

10 h

1

2

1. Basal measurement
2. SP (10 µg/kg)

Measurements at 10 h made at 10 minute intervals
Fig. 2 (B)
Fig. 3 (A)

Time: 0 h

Cisplatin (5 mg/kg)

1. Basal measurement
2. SP (10 µg/kg)
3. Palonosetron (30, 100 & 300 µg/kg)
4. SP

Measurements at 10 h made at 10 minute intervals
Fig. 3 (B)
Fig. 4 (A)

Time: 0 h

Cisplatin

10 h

1 2 3 4

1. Basal measurement
2. SP
3. O, G or P
4. SP

Measurements at 10 h
made at 10 - 30 minute intervals

SP: 10 µg/kg; Ondansetron (O): 38 mg/kg; Granisetron (G): 840 µg/kg
Palonosetron (P): 300 µg/kg; Cisplatin: 5 mg/kg
Fig. 4 (B)
Fig. 5 (A)

- 0.5 h 0 h 5 h 10 h

1: Cisplatin
2: O, G or P
3: Cisplatin
4: Cisplatin

1: SP
2: SP
3: SP
4: O, G or P + SP

Measurements at 10 h

SP: 10 µg/kg; Ondansetron (O): 38 mg/kg; Granisetron (G): 840 µg/kg; Palonosetron (P): 300 µg/kg; Cisplatin: 5 mg/kg
Fig. 5 (B)

30 min before cis 5 h after cis 10 h after cis

Cisplatin Ondansetron Granisetron Palonosetron Ondansetron Granisetron Palonosetron Ondansetron Granisetron Palonosetron

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