

# The Antiemetic 5-HT<sub>3</sub> Receptor Antagonist Palonosetron Inhibits Substance P-Mediated Responses In Vitro and In Vivo<sup>S</sup>

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## ABSTRACT

Palonosetron is the only 5-HT<sub>3</sub> receptor antagonist approved for the treatment of delayed chemotherapy-induced nausea and vomiting (CINV) in moderately emetogenic chemotherapy. Accumulating evidence suggests that substance P (SP), the endogenous ligand acting preferentially on neurokinin-1 (NK-1) receptors, not serotonin (5-HT), is the dominant mediator of delayed emesis. However, palonosetron does not bind to the NK-1 receptor. Recent data have revealed cross-talk between the NK-1 and 5HT<sub>3</sub> receptor signaling pathways; we postulated that if palonosetron differentially inhibited NK-1/5-HT<sub>3</sub> cross-talk, 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. After preincubation with palonosetron, but not ondansetron or granisetron, the serotonin enhancement of the SP response was inhibited. Rats were treated with cisplatin and either palonosetron, granisetron, or ondansetron. At various times after dosing, single neuronal recordings from nodose ganglia were collected after 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-HT<sub>3</sub> 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-HT<sub>3</sub> receptor antagonists are most efficacious against acute emesis (0–24 h after chemotherapy administration), whereas neurokinin-1 (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-HT<sub>3</sub> 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, because palonosetron does not bind to NK-1 receptors (Wong et al., 1995).

Receptor cross-talk, defined as activation of one receptor by its ligand affecting cellular responses to another receptor system, has been well described. Recent reports in the literature have shown that there is cross-talk between NK-1 and 5-HT<sub>3</sub> receptor signaling pathways. For example, substance

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**ABBREVIATIONS:** CINV, chemotherapy-induced nausea and vomiting; 5-HT, serotonin; NK-1, neurokinin-1; SP, substance P; AM, acetoxymethyl.

P (SP), an agonist at the NK-1 receptor, was shown to potentiate 5-HT<sub>3</sub> receptor-mediated inward current in rat trigeminal ganglion neurons (Hu et al., 2004). In separate studies, 5-HT<sub>3</sub> 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 cross-talk raises the interesting possibility that palonosetron's unique efficacy in delayed emesis could be caused by differential inhibition of the 5-HT<sub>3</sub>/NK-1 receptor cross-talk. 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-HT<sub>3</sub> receptor antagonists. The results suggest palonosetron differentially inhibits 5-HT<sub>3</sub>/NK-1 receptor signaling cross-talk and provide a rationale to explain palonosetron's unique efficacy against delayed emesis in the clinic.

## Materials and Methods

**Calcium-Ion Release Measurements in NG108-15 Cells.** NG108-15 cells, known to express both 5-HT<sub>3</sub> and NK-1 receptors, were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with a mixture of sodium hypoxanthine, aminopterin, and thymidine, 10% heat-inactivated fetal bovine serum, and 2 mM glutamine to 90% confluence. 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 on  $K_d$  values described previously 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 an additional hour to allow for dissociation of antagonists still bound to the cell. Next, cell media were replaced with isosmotic HEPES buffer (20 mM, pH 7.4, 130 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, and 2 mM CaCl<sub>2</sub>) containing 2  $\mu$ M 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 caused by SP alone or SP plus serotonin ( $10^{-7}$  M final concentration added immediately before measurement) was made by using a fluorometric imaging plate reader.

**Animal Preparation.** Studies were conducted in accordance with the Declaration of Helsinki and 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  $22 \pm 2^\circ\text{C}$ , and relative humidity was set at  $55 \pm 10\%$ . Artificial lighting was provided for 24 h with a cycle of 12-h light/12-h dark (light 7 AM–7 PM). Animals had free access to food and water; they were anesthetized with an intraperitoneal injection of a mixture of  $\alpha$ -chloralose (80 mg/kg) and urethane (800 mg/kg). The anesthetic was supplemented every 3.5 h with an intravenous dose of  $\frac{1}{4}$  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 cm<sup>3</sup> tidal

volumes). A midline abdominal incision exposed the abdominal vago, 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  $\mu$ m) around the anterior and posterior trunks, approximately 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 intraperitoneally, SP (10  $\mu$ g/kg) was given by intra-arterial injection, and antagonists were administered by intravenous 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  $\mu$ g/100- $\mu$ l solution. Total dose of 840  $\mu$ g/kg for granisetron was administered over 10 min in 24  $\mu$ g/100- $\mu$ l solution. Total doses of 30, 100, and 300  $\mu$ g/kg for palonosetron were administered over 10 min in 0.9, 3, and 9  $\mu$ g/100- $\mu$ l solutions, respectively.

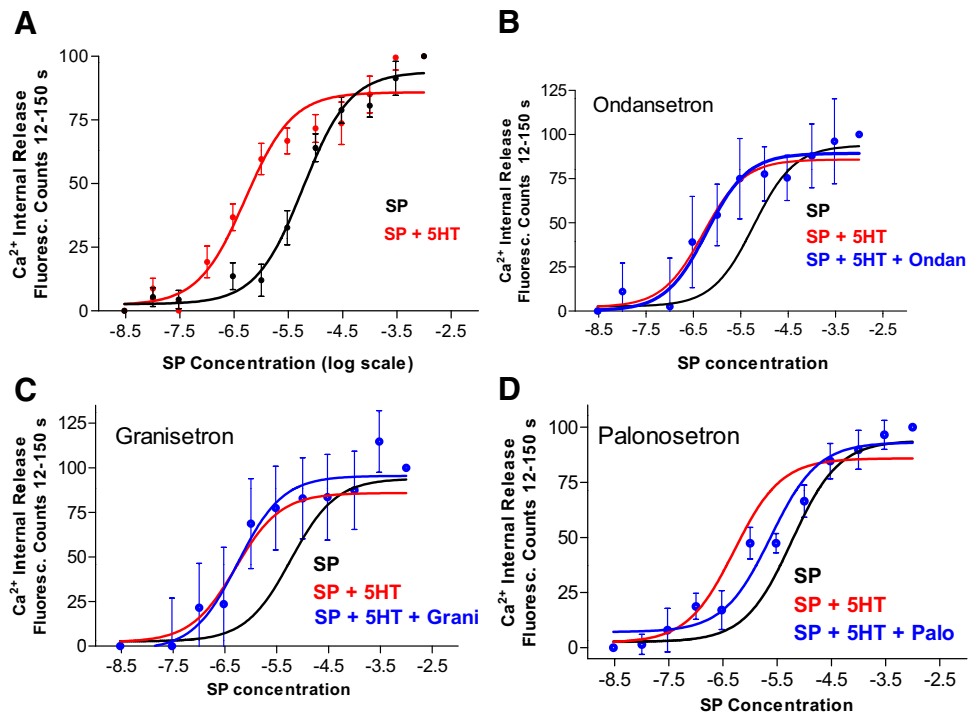
**Recording of Single Nodose Neuronal Activity.** Rats were placed in a small animal stereotaxic frame (David Kopf Instruments, Tujunga, CA). Body temperature was maintained with a heating pad. The right nodose ganglion was exposed by a short dorsal approach. Using an operating microscope, the ganglion 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  $\mu$ m in diameter with a resistance of 50 to 70 M $\Omega$ . 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 Figs. 2 to 5 are the average from 12 to 42 measurements made on isolated neurons obtained from 7 to 26 rats.

**Statistical Analysis.** Prism (GraphPad Software Inc., San Diego, CA) was used to obtain EC<sub>50</sub> values in in vitro experiments (sigmoidal dose-response variable slope). Errors correspond to S.E.M. of at least eight 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 seven 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 depended on SP concentration with an EC<sub>50</sub> of  $6.7 \pm 0.97 \mu\text{M}$ ; when serotonin ( $10^{-7}$  M) was present the EC<sub>50</sub> shifted 10-fold to the left to  $0.62 \pm 0.09 \mu\text{M}$  (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<sub>50</sub> values were the same as those obtained with SP plus serotonin (Fig. 1, B and C; Table 1). In contrast, preincubation with palonosetron inhibited the serotonin enhancement of the SP response; the EC<sub>50</sub> shifted 6-fold to the right to  $3.7 \pm 0.84 \mu\text{M}$ .  $p < 0.001$  compared with the EC<sub>50</sub> 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 after intra-arterial administration of vehicle was negligible (<1 impulse/10s). When SP was administered at



**Fig. 1.** The effect of 5-HT<sub>3</sub> 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 1 h internal calcium release was measured upon the addition of  $\pm$  serotonin ( $10^{-7}$  M). B, ondansetron (Ondan; 30 nM) was preincubated 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 SP + 5HT in A are shown for reference. C and D, same as B except granisetron (Grani; 5 nM; C) and palonosetron (Palo; 1 nM; D) were used. Error bars correspond to  $\pm$  S.E.M.; error bars in B–D for the SP and SP + 5HT traces are the same as in A. Each EC<sub>50</sub> curve corresponds to the average of at least eight independent determinations.

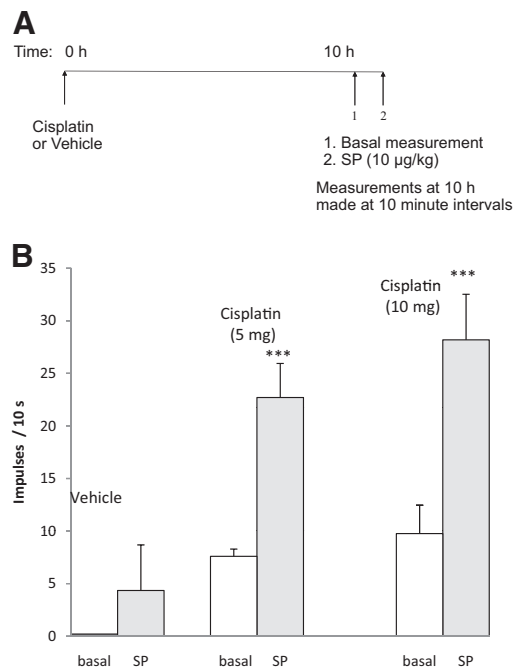
**TABLE 1**  
Effect of 5-HT<sub>3</sub> receptor antagonists on serotonin activation of SP response in NG-108-15 cells

EC<sub>50</sub> values correspond to the results shown in Fig. 1. GraphPad Prism was used to obtain EC<sub>50</sub> values (sigmoidal dose-response variable slope). Errors correspond to  $\pm$  S.E.M. Data are the average of at least eight independent determinations. EC<sub>50</sub> for SP + 5-HT + palonosetron was statistically different with  $P < 0.001$  compared with SP + 5-HT by using Student's *t* test.

Treatment	EC <sub>50</sub> $\pm$ S.E.M.
	$\mu$ M
SP	6.7 $\pm$ 0.97
SP + 5-HT	0.62 $\pm$ 0.09
SP + 5-HT + ondansetron	0.66 $\pm$ 0.17
SP + 5-HT + granisetron	0.57 $\pm$ 0.10
SP + 5-HT + palonosetron	3.7 $\pm$ 0.84

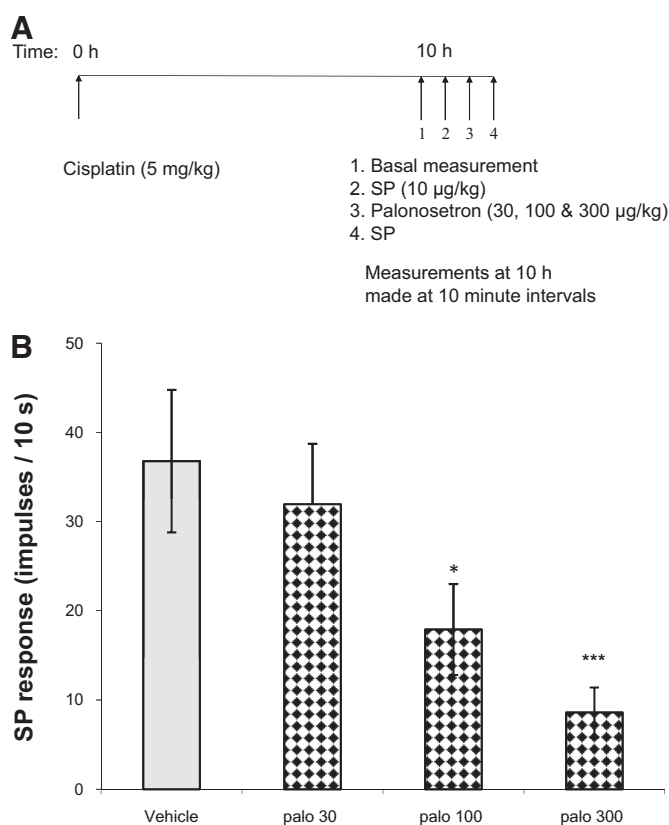
10  $\mu$ g/kg via intra-arterial injection neuronal activity was increased, although variable, to  $4 \pm 4$  impulses/10 s. When measurement of the SP response was made 10 h after intraperitoneal administration of 5 mg/kg i.p. cisplatin (Fig. 2A), SP responses increased to  $23 \pm 3$  impulses/10 s. When the dose of cisplatin was increased to 10 mg/kg, both basal and SP responses were similar within experimental error to when 5 mg/kg cisplatin was used (Fig. 2B). In short, there was an approximately 6-fold increase in the neuronal response to SP in the presence of 5 to 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; 10 h later, single nodose ganglia neurons were isolated and four electrophysiological recordings were made at 10-min intervals (Fig. 3A). First, a basal reading was taken. Second, the neuronal response to SP (10  $\mu$ g/kg intra-arterial injection) was measured. Third, the neuronal response to palonosetron (intravenous infusion of 30, 100, and 300  $\mu$ g/kg) was mea-



**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. Before 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 seven rats (\*\*\*,  $p < 0.001$ ; compared with no cisplatin). Error bars correspond to  $\pm$  S.E.M.

sured. The fourth and final recording measured neuronal responses after the administration of SP (10  $\mu$ g/kg intra-arterial injection). The first three responses were similar throughout the experiment: basal readings were  $7.5 \pm 3$



**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 four different measurements of neuronal activity were made at 10-min intervals: 1) basal measurement, 2) response to SP, 3) response to palonosetron alone; 4) response to SP after palonosetron administration. B, neuronal response to SP after palonosetron (palo) administration at 30, 100, and 300  $\mu\text{g}/\text{kg}$ ; only the fourth measurement at each concentration is shown. Results are the average of at least 12 independent neuronal measurements from at least seven rats (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; compared with vehicle). Error bars correspond to  $\pm$  S.E.M.

impulses/10 s. SP increased the neuronal activity to  $38 \pm 4$  impulses/10 s, and palonosetron alone at 30, 100, or 300  $\mu\text{g}/\text{kg}$  did not have an effect on either basal or SP-induced responses. However, when SP was administered after palonosetron, the neuronal responses dose-dependently decreased to  $32 \pm 4$  (SP + 30  $\mu\text{g}/\text{kg}$  palonosetron),  $18 \pm 2$  (SP + 100  $\mu\text{g}/\text{kg}$  palonosetron), and  $9 \pm 3$  (SP + 300  $\mu\text{g}/\text{kg}$  palonosetron) impulses/10 s (Fig. 3B).

**Ondansetron and Granisetron Had No Effect on the Cisplatin Enhancement of the SP Response in Nodose Ganglia.** Because palonosetron exhibited inhibition of the cisplatin-induced SP response, we explored more thoroughly whether the effect was common to other 5-HT<sub>3</sub> receptor antagonists such as granisetron and ondansetron. In an attempt to replicate the results with ondansetron and granisetron, experimental doses that accounted for their differences in clinical dose versus 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  $\mu\text{g}/\text{kg} \times 128 = 38$  mg/kg). Likewise, 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  $\mu\text{g}/\text{kg} \times 2.8 = 840$   $\mu\text{g}/\text{kg}$ ).

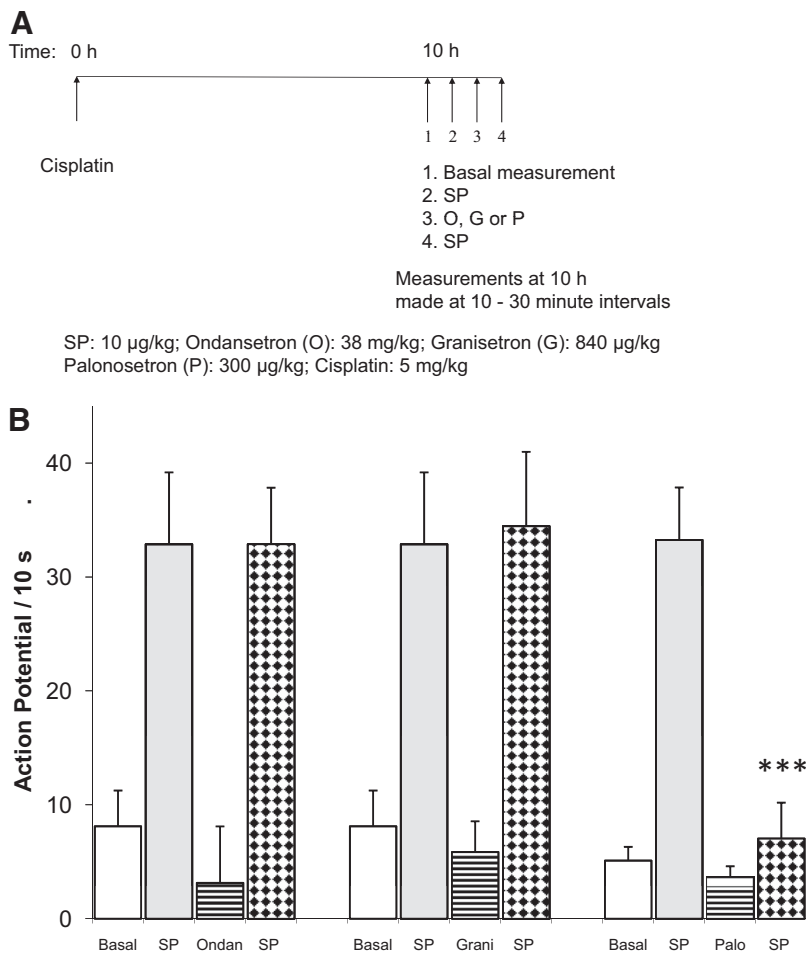
The doses used for ondansetron and granisetron in these studies turned out to be in excess of their active doses reported in previous efficacy studies in animals (Eglen et al., 1995; Rudd and Naylor, 1996; Endo et al., 1999; Rudd et al., 2002). Vagal nerve activity was measured in animals 10 h after 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  $8 \pm 3$  and  $33 \pm 6$  impulses/10 s, respectively. Neither ondansetron (38 mg/kg i.v.) nor granisetron (840  $\mu\text{g}/\text{kg}$  i.v.) had an effect on the basal response. When SP was administered after ondansetron or granisetron, the SP response was not affected. This lack of effect was in stark contrast to more than 70% inhibition of the SP response after palonosetron administration (Fig. 4B).

**Palonosetron's Inhibition of the Cisplatin-Induced SP Response Was Time-Dependent.** One major consideration in these experiments and in the clinic is the time of administration of 5-HT<sub>3</sub> 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  $32 \pm 4$  impulses/10 s. When ondansetron (38 mg/kg i.v.) or granisetron (840  $\mu\text{g}/\text{kg}$  i.v.) was 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  $\mu\text{g}/\text{kg}$  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 (Fig. 5B).

## Discussion

5-HT<sub>3</sub> and NK-1 receptor antagonists are common therapy to help prevent CINV. In general, 5-HT<sub>3</sub> 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-HT<sub>3</sub> receptor antagonist that is labeled for the treatment of delayed emesis in moderately emetogenic chemotherapy. 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-HT<sub>3</sub> receptor. Palonosetron exhibits allosteric binding and positive cooperativity (Rojas et al., 2008) and triggers receptor internalization and long-term inhibition of receptor function (Rojas et al., 2010), attributes not shared by other 5HT<sub>3</sub> receptor antagonists.

Various reports in the literature have shown that there is cross-talk between NK-1 and 5-HT<sub>3</sub> receptor signaling path-



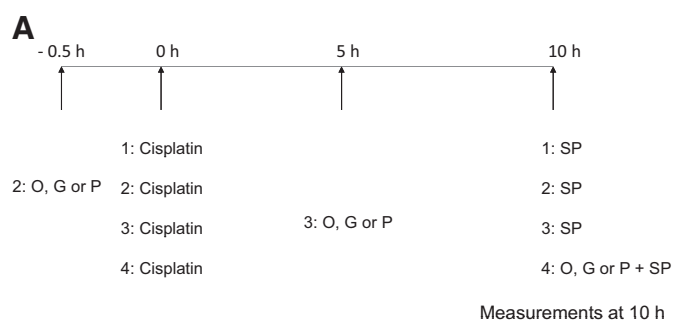
**Fig. 4.** Effects of 5-HT<sub>3</sub> receptor antagonists on the cisplatin-induced neuronal response to SP. A, experimental protocol. Cisplatin was given and 10 h later four different measurements of neuronal activity were made at 10- to 30-min intervals: 1) basal measurement, 2) response to SP, 3) effect on baseline of ondansetron, granisetron or palonosetron, and 4) response to SP after ondansetron, granisetron, or palonosetron. B, set of four measurements for each antagonist [ondansetron (Ondan), granisetron (Grani), or palonosetron (Palo)] as described in A. Results are the average of at least 12 independent neuronal measurements from at least seven rats (\*\*\*,  $p < 0.001$  compared with SP). Error bars correspond to  $\pm$  S.E.M.

ways (Hu et al., 2004). SP, a known agonist at the NK-1 receptor, was shown to potentiate 5-HT<sub>3</sub> receptor-mediated inward current in rat trigeminal ganglion neurons. Potentiation of 5-HT<sub>3</sub> receptor current through SP is thought to involve second-messenger signaling that culminates in protein kinase C activation (Hu et al., 2004). In addition, 5-HT<sub>3</sub> 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 caused by differential inhibition of the 5-HT<sub>3</sub>/NK-1 receptor cross-talk. In the present work, we evaluated the effect of palonosetron, granisetron, and ondansetron on SP-induced responses in vitro and in vivo.

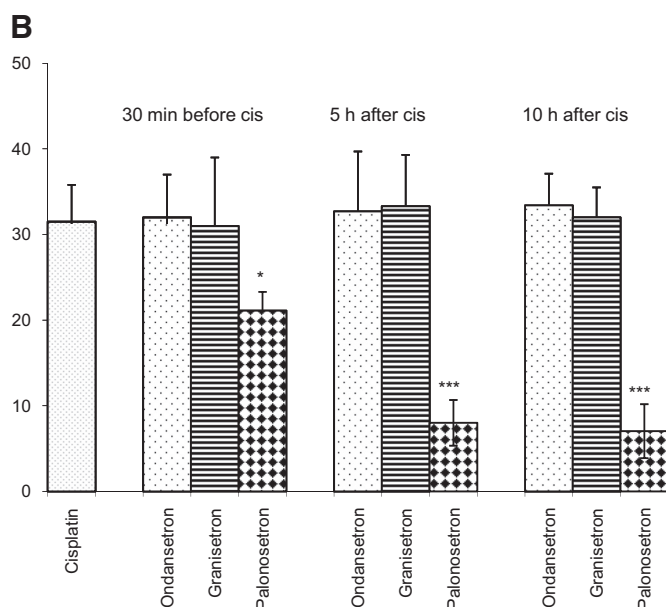
NG108-15 cells were used in the in vitro studies because they are known to express both 5-HT<sub>3</sub> and NK-1 receptors (Reiser and Hamprecht, 1989; Emerit et al., 1993). Previous studies have also shown that SP, acting through the NK-1 receptor, stimulates an increase of intracellular calcium ions 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 depended on SP concentration ( $EC_{50} = 6.7 \pm 0.97 \mu\text{M}$ ). In addition, serotonin, at a subthreshold concentration ( $10^{-7}\text{M}$ ), where it did not elicit calcium-ion mobilization by

itself, induced a 10-fold potency increase of the response to SP alone ( $EC_{50} = 0.62 \pm 0.09 \mu\text{M}$ ; Fig. 1A; Table 1). This result was in agreement with previous findings pointing to interactions between the signaling of 5-HT<sub>3</sub> and NK-1 receptors in rat trigeminal ganglion neurons, where serotonin potentiation of inward current was enhanced by SP preapplication (Hu et al., 2004).

Further, NG108-15 cells provided a simple in vitro system to evaluate the potential differential inhibition of 5-HT<sub>3</sub>/NK1 receptor cross-talk by 5-HT<sub>3</sub> receptor antagonists. We have shown that palonosetron triggers long-term 5-HT<sub>3</sub> receptor internalization, whereas ondansetron and granisetron exhibit simple receptor blockade (Rojas et al., 2010). The purpose of these in vitro experiments was to determine whether preincubation with the 5-HT<sub>3</sub> antagonists, followed by their complete removal from the media, could have a persistent downstream effect on SP function. In other words, could 5-HT<sub>3</sub> receptor internalization affect 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  $IC_{50}$  values needed to inhibit serotonin-induced calcium-ion influx (Supplemental Methods and Supplemental Fig. 1, A-C;  $IC_{50}$ s 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



SP: 10  $\mu\text{g}/\text{kg}$ ; Ondansetron (O): 38 mg/kg; Granisetron (G): 840  $\mu\text{g}/\text{kg}$ ;  
Palonosetron (P): 300  $\mu\text{g}/\text{kg}$ ; Cisplatin: 5 mg/kg



**Fig. 5.** Effect of time of administration of 5-HT<sub>3</sub> 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 (cis). Results are the average of at least 12 independent neuronal measurements from at least seven rats (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; compared with cisplatin). Error bars correspond to  $\pm$  S.E.M.

through extensive cell washing, the serotonin effect on the response to SP was inhibited 6-fold (rightward shift of the EC<sub>50</sub> 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. 1, C and D; Table 1). Cell washing to remove the antagonists from the media took into account their respective half-lives of dissociation from 5-HT<sub>3</sub> 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 probably are caused by palonosetron's distinctive ability to induce 5-HT<sub>3</sub> receptor internal-

ization and cause long-term reduction in 5-HT<sub>3</sub> receptor density on the cell surface. Previously, we have shown palonosetron rapidly partitions inside cells along with the 5-HT<sub>3</sub> receptor during antagonist preincubation; once inside the cell, palonosetron does not reappear in the extracellular milieu for at least 2 h. It is noteworthy that palonosetron's internalization depends on the presence of the 5-HT<sub>3</sub> receptor, because palonosetron was shown not to partition into cells that do not express the 5-HT<sub>3</sub> receptor (Rojas et al., 2010). These data suggest that palonosetron's unique effect on inhibition of serotonin-induced SP activation is a 5-HT<sub>3</sub> receptor-mediated difference, not a cell permeability difference.

To determine whether 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-HT<sub>3</sub> 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 enterochromaffin cells, which in turn activates 5-HT<sub>3</sub> receptors located on the surface of vagal afferents. Consistent with previous data showing that 5-HT<sub>3</sub> 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).

Because cisplatin potentiated the SP response in nodose ganglia, we explored the possibility that 5-HT<sub>3</sub> 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 (Figs. 3 and 4). Neither ondansetron nor granisetron had an effect. It is noteworthy that 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 used 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-HT<sub>3</sub> 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 et al. (2001) reported that granisetron inhibits the SP response under acute conditions in vivo. However, our in vivo study was different in that it was designed to explore the effect of 5-HT<sub>3</sub> 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-HT<sub>3</sub> receptor antagonists on this enhancement. Under these conditions only palonosetron inhibited the enhanced interaction of SP and serotonin in vagal afferent neurons. These differences probably are caused by palonosetron-triggered 5HT<sub>3</sub> receptor internalization that is unique and would be predicted to provide more persistent 5HT<sub>3</sub> receptor functional inhibition compared with simple

binding followed by dissociation at the receptor on the cell surface as occurs with ondansetron and granisetron.

Because 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 when antagonists were given 30 min before cisplatin and 5 h after cisplatin. When ondansetron or granisetron was used, no inhibition of the cisplatin effect was observed at either time point. In contrast, when palonosetron was administered 30 min before cisplatin or 5 h after cisplatin and the effect on SP activity was measured 10 h later  $33 \pm 7$  and  $70 \pm 8\%$  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 intravenous administration (Supplemental Data, Palonosetron Rat PK Report), 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 probably are not caused by differences in pharmacokinetics.

In summary, palonosetron uniquely inhibits cross-talk between the 5-HT<sub>3</sub> and NK-1 receptor pathways in a dose- and time-dependent fashion. Our results are consistent with previous data showing that palonosetron exhibits distinct pharmacology versus the older 5-HT<sub>3</sub> receptor antagonists and provide a rationale for the unique efficacy observed with palonosetron in delayed CINV in the clinic.

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