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Inhibition of substance P-mediated responses in NG108-15 cells by netupitant and palonosetron exhibit synergistic effects

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ABSTRACT

Netupitant is a potent and selective NK_1 receptor antagonist under development in combination with a fixed dose of palonosetron for the prevention of chemotherapy induced nausea and vomiting. Palonosetron is a 5-HT_3 receptor antagonist approved for both the prevention of acute and delayed chemotherapy induced nausea and vomiting after moderately emetogenic chemotherapy. Accumulating evidence suggests that substance P (SP), a ligand acting largely on tachykinin (NK_1) receptors, is the dominant mediator of delayed emesis. Interestingly, palonosetron does not bind to the NK_1 receptor so that the mechanism behind palonosetron's unique efficacy against delayed emesis is not clear. Palonosetron exhibits a distinct ability among 5-HT_3 receptor antagonists to inhibit crosstalk between NK_1 and 5-HT_3 receptor signaling pathways. The objective of the current work was to determine if palonosetron's ability to inhibit receptor signaling crosstalk would influence netupitant's inhibition of the SP-mediated response when the two drugs are dosed together. We first studied the inhibition of SP-induced Ca^{2+} mobilization in NG108-15 cells by palonosetron, ondansetron and granisetron. Unexpectedly, in the absence of serotonin, palonosetron inhibited the SP-mediated dose response 15-fold; ondansetron and granisetron had no effect. Netupitant also dose-dependently inhibited the SP response as expected from an NK1 receptor antagonist. Importantly, when both palonosetron and netupitant were present, they exhibited an enhanced inhibition of the SP response compared to either of the two antagonists alone. The results further confirm palonosetron's unique pharmacology among 5-HT_3 receptor antagonists and suggest that it can enhance the prevention of delayed emesis provided by NK_1 receptor antagonists.

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1. Introduction

Current therapy for the treatment of chemotherapy induced nausea and vomiting includes the use of both 5-HT_3 and NK_1 receptor antagonists along with dexamethasone (Basch et al., 2011; Ettinger et al., 2011; Roila et al., 2010). Acute emesis has been largely associated with activation of 5-HT_3 receptors by serotonin while delayed emesis is thought to occur mainly through the activation of NK_1 receptors by substance P (SP) (Hesketh et al., 2003). While treatment of acute emesis was largely resolved with the introduction of 5-HT_3 receptor antagonists in the 1990s, nausea and delayed emesis are lingering problems (Feyer and Jordan, 2011; Oo and Hesketh, 2005). The introduction of NK_1 receptor antagonists has been shown to improve overall antiemetic efficacy including delayed emesis when used along with 5-HT_3 receptor antagonists and dexamethasone (Darmani and Ray, 2009; Feyer and Jordan, 2011; Hesketh et al., 2003). Even though a majority of patients are fully protected against chemotherapy induced nausea and vomiting by the use of these therapies, there are still a significant number of patients that experience nausea and delayed emesis, especially following highly or moderately emetogenic chemotherapies (Feyer and Jordan, 2011). Netupitant is a potent and selective NK_1 receptor antagonist currently under development in combination with a fixed dose of palonosetron for the prevention of chemotherapy induced nausea and vomiting. Palonosetron is the only 5-HT_3 receptor antagonist that has been found to be effective in both acute and delayed chemotherapy induced nausea and vomiting after moderate emetogenic chemotherapy (Aapro et al., 2006; Eisenberg et al., 2003; Gralla et al., 2003; Saito et al., 2009). Since palonosetron does not bind to the NK_1 receptor, the mechanism behind palonosetron's unique efficacy among 5-HT_3 receptor antagonists against nausea and delayed emesis is

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obscure. Recent mechanism of action studies showed that palonosetron could inhibit SP mediated responses in vitro and in vivo possibly as a result of inhibition of 5-HT_3/NK_1 receptor crosstalk (Rojas et al., 2010b). It is not clear however, if palonosetron's effect on 5-HT_3/NK_1 receptor crosstalk would enhance or negate the inhibition of the SP response when used in combination with NK_1 antagonists. In the present work, we used NG108-15 cells known to express both the 5-HT_3 and NK_1 receptors (Emerit et al., 1993; Reiser and Hamprecht, 1989), to study the inhibition of the SP response by netupitant and palonosetron. We report that netupitant and palonosetron exhibit a synergistic effect in the prevention of the SP-mediated response in these cells.

2. Materials and methods

2.1. Calcium-ion release measurements in NG108-15 cells

NG108-15 cells were grown to 95% confluence in high-glucose Dulbecco's modified Eagle's medium. Medium was supplemented with a mixture of sodium hypoxanthine, aminopterin, and thymidine, 10% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin (100 units), streptomycin (100 μg), and amphotericin B (0.25 μg). All media exchanges were preceded by a 2–5 min plate spin at 168 × g, to prevent loosely adherent cells from coming off the plate surface. When determining the effect of 5-HT_3 receptor antagonists on the SP response, cells were preincubated for 1 h at 37 °C with either growth media alone (control) or media containing palonosetron (6 nM), ondansetron (300 nM) and granisetron (50 nM). Antagonist concentrations were at least 30-fold the K_d value to ensure receptor saturation (Rojas et al., 2010b). After preincubation, antagonists were removed and cells were rinsed with growth media alone for an additional hour to allow for dissociation of antagonists still bound to receptor. Cell media were then replaced with isosmotic HEPES buffer (pH 7.4, 20 mM) containing NaCl (130 mM), KCl (2 mM), $MgCl_2$ (1 mM) $CaCl_2$ (2 mM), Fluo-4 acetoxymethyl (AM) ester (2 μM), pluronic acid (0.04%) and SP at various concentrations in the 3 nM to 1 mM range. The final incubation lasted for 1 h at 37 °C. Pluronic acid was added as a nonionic surfactant to sequester the AM ester molecules into micelles for cellular uptake. Calcium-ion release was measured for 3 min using a fluorimetric imaging plate reader (FLIPR 1 system), utilizing a Detector Controller (Princeton Instruments, Inc.) and a Sapphire CDRH-HP air-cooled laser at 250 mW, 488 nm excitation (Coherent Inc., Santa Clara, CA).

Experimental protocol was the same when determining the response to change of concentration of netupitant, palonosetron, or netupitant plus palonosetron; in this case, various concentrations of antagonist(s) were used during preincubation. Similarly, SP was replaced by [Sar^9, $Met(O_2)^{11}$] Sub-P, GR-64349, or Senktide (0.6–200 μM) when determining the effects of selective NK_1, NK_2 and NK_3 agonists respectively.

2.2. Statistical analysis

FLIPR Control Software for Windows NT (Version 1.13, Molecular Devices Inc., Sunnyvale, CA) was employed to record the measurements. The max–min signal values generated between 12 and 150 s were subsequently analyzed. Data were normalized to SP control values for baseline and maximal response and a nonlinear regression variable slope analysis using Prism (GraphPad Software Inc, San Diego, CA) was used to obtain EC_{50} values of normalized response vs. log of inhibitor concentration. Error bars correspond to standard error of the mean (S.E.M.).

3. Results

3.1. Palonosetron inhibited the SP response in the absence of serotonin

When cells were preincubated with a saturating concentration of palonosetron (6 nM) in the absence of serotonin followed by media changes to remove antagonist remaining on the cell surface, the SP-induced response was significantly inhibited. The SP response after preincubation with palonosetron exhibited an EC_{50} of 30 ± 6 μM which corresponded to a 15-fold shift to the right compared to the SP response in control cells i.e., when there was no preincubation with palonosetron (EC_{50}=2 ± 0.2 μM) (Fig. 1). In contrast, the SP response was not affected by preincubation with saturating concentrations of granisetron (50 nM) or ondansetron (300 nM) (Fig. 1).

3.2. Inhibition of SP response by netupitant and palonosetron is synergistic

In order to study the effect of palonosetron on the inhibition of the SP response by an NK_1 receptor antagonist (netupitant), we first determined the effect of each individual agent separately on the SP response at various concentrations. The EC_{50} of the SP response was not changed by netupitant at 1 nM, it gradually shifted to the right at 3 and 10 nM and there was maximal inhibition at 30 nM (Table 1 and Fig. 2A). Similarly, palonosetron exhibited a threshold concentration (0.2 nM) at which there was no effect on the SP response. Palonosetron increasingly inhibited the SP response at 0.6 and 2 nM and had maximal inhibitory effect at 6 nM (Table 1 and Fig. 2B).

We then determined the effect on the SP response when both palonosetron and netupitant were present at threshold concentrations, i.e., concentrations at which neither antagonist inhibited the SP response: netupitant at 1 nM and palonosetron at 0.2 nM. When both antagonists were present at threshold concentrations there was an approximately 60-fold shift in EC_{50} of the SP response when compared to the EC_{50} in the presence of each antagonist alone (Table 1, Fig. 2C).

In the next set of experiments, we determined the effect on the SP response when both palonosetron and netupitant were present

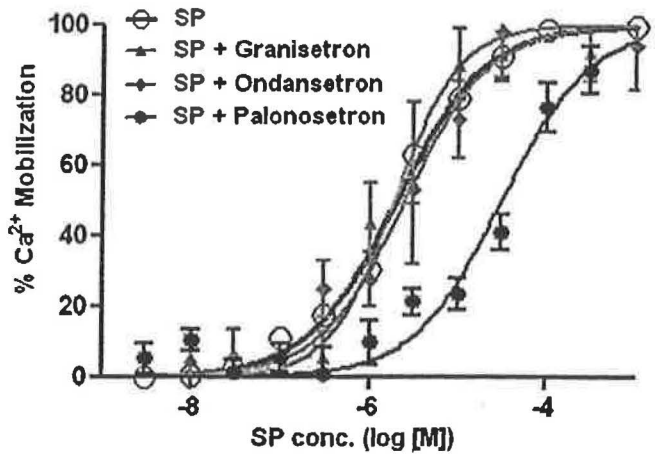

Fig. 1. Effect of 5-HT_3 receptor antagonists on SP induced calcium-ion mobilization in NG108-15 cells. Cells were pre-incubated with saturating concentrations (≥30-fold K_d) of granisetron (50 nM), ondansetron (300 nM) or palonosetron (10 nM) for 1 h at 37 °C. Subsequent to complete removal of antagonists, the cells were incubated with SP at different concentrations before measurement of calcium-ion mobilization (Materials and Methods). EC_{50} values for SP (n=38), SP+ondansetron (n=4) and SP+granisetron (n=3) were all 2 μM. EC_{50} value for SP+palonosetron was 30 ± 6 μM (n=5). Error bars correspond to ± S.E.M.

Table 1
Inhibition of SP response in NG108-15 cells by netupitant (netu) and palonosetron (palo).

SP+Antagonist(s)	EC_{50} (μM) ± S.E.M.	*n*
SP only	2 ± 0.2	38
Plus netu (1 nM)	2 ± 0.3	3
Plus netu (3 nM)	5 ± 3	3
Plus netu (10 nM)	10 ± 0.9	3
Plus netu (30 nM)	40 ± 10[a]	6
Plus palo (0.2 nM)	2 ± 0.1	3
Plus palo (0.6 nM)	3 ± 0.6	3
Plus palo (2 nM)	10 ± 2	3
Plus palo (6 nM)	30 ± 6[a]	5
Plus net (1 nM) plus palo (0.2 nM)	120 ± 20[b]	7
Plus net (30 nM) plus palo (6 nM)	970 ± 150[c]	5

Cells were pre-incubated with netupitant, palonosetron or combinations of palonosetron plus netupitant at various concentrations as indicated for 1 h at 37 °C. After antagonist(s) removal, cells were incubated at 37 °C for 1 h with various SP concentrations, followed by measurement of calcium-ion mobilization. SP only had $n=38$ because it was used as control in all experiments. When using antagonists, data were the average of $n=3-7$ determinations as indicated. GraphPad Prism was used to obtain EC_{50} values (Materials and Methods). The traces with the experimental points that generated the EC_{50} values are given in Fig. 2.

[a] $P<0.001$ compared to SP only.
[b] $P<0.05$ compared to netupitant alone at 1 nM or palo alone at 0.2 nM.
[c] $P<0.001$ compared to netupitant alone at 30 nM or to palo alone at 6 nM.

at concentrations where maximal inhibition was observed with each antagonist: netupitant at 30 nM and palonosetron at 6 nM. The EC_{50} of the dose response under these conditions was 970 ± 150 μM, representing a 22-fold shift with respect to maximal inhibition by netupitant ($P<0.001$) and a 30-fold shift with respect to maximal inhibition by palonosetron ($P<0.001$) (Table 1, Fig. 2D). In cases where maximal activity was not reached even at 1 mM SP, the EC_{50}s represent the concentration of SP required to obtain 50% of the control response rather than 50% of maximal activity. There was a decrease in the maximum response when both palonosetron and netupitant were used (R_{max}) suggesting that fewer receptors became available. The results showed a synergistic effect on the inhibition of the SP response both when palonosetron and netupitant were present at threshold and when using maximal inhibitory concentrations.

3.3. *Inhibition of the SP-response by netupitant and palonosetron occurs selectively through inhibition of the NK_1 receptor signaling pathway*

Experiments with selective NK_1 ([Sar[9], Met(O_2)[11]]-Substance P), NK_2 (GR 64349) and NK_3 (Senktide) receptor agonists exhibited calcium-ion mobilization in a dose-response manner indicating that these three receptors are found in NG108-15 cells.

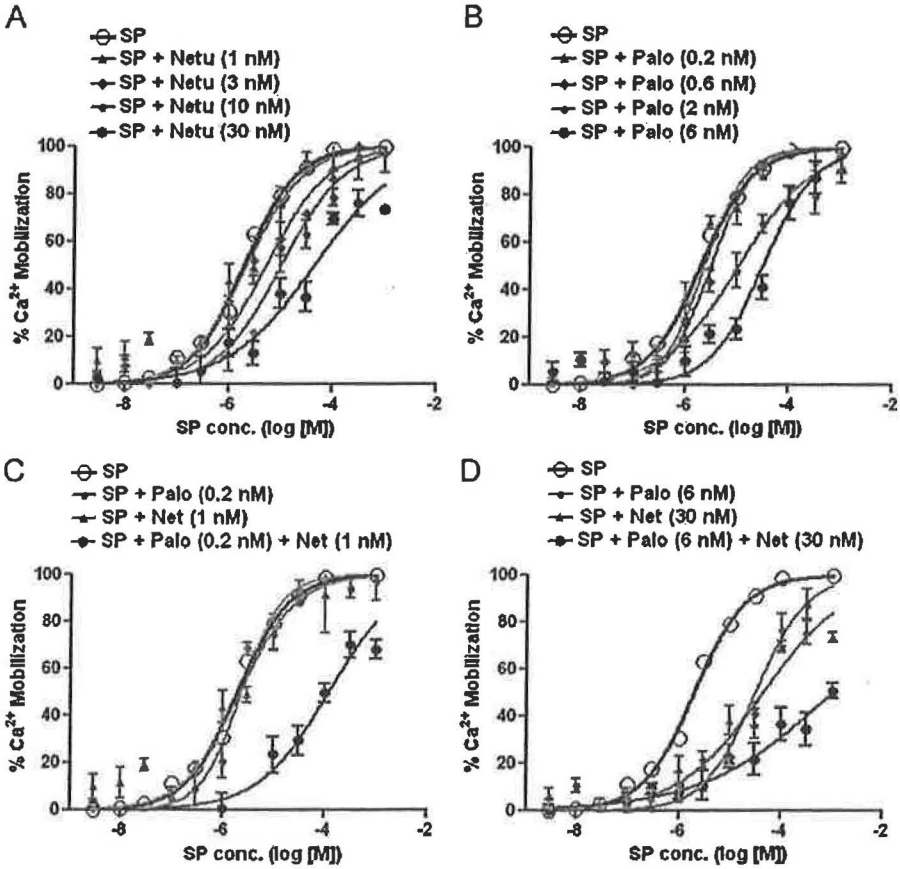

Fig. 2. Effect on SP response in NG108-15 cells when using palonosetron alone, netupitant alone and palonosetron plus netupitant. Cells were pre-incubated with netupitant, palonosetron or a combination of palonosetron plus netupitant. After antagonist(s) removal, cells were incubated at 37 °C for 1 h with various SP concentrations, followed by measurement of calcium-ion mobilization. (A) Netupitant alone. (B) Palonosetron alone. (C) Threshold concentrations of netupitant (1 nM) and palonosetron (0.2 nM) alone and in combination. Threshold concentrations were concentrations at which no inhibition of the SP response was observed when each antagonist was used alone. (D) Maximal concentrations of netupitant (30 nM) and palonosetron (6 nM) alone and in combination. Maximal concentrations were concentrations at which maximal inhibition of the SP response was observed when each antagonist was used alone. EC_{50}s and *n* values for each trace are listed in Table 1 In cases where maximal activity was not reached even at an SP concentration of 1 mM, the EC_{50}s represent the SP concentration required to obtain 50% of the control response rather than 50% of maximal activity. Error bars correspond to ± S.E.M.

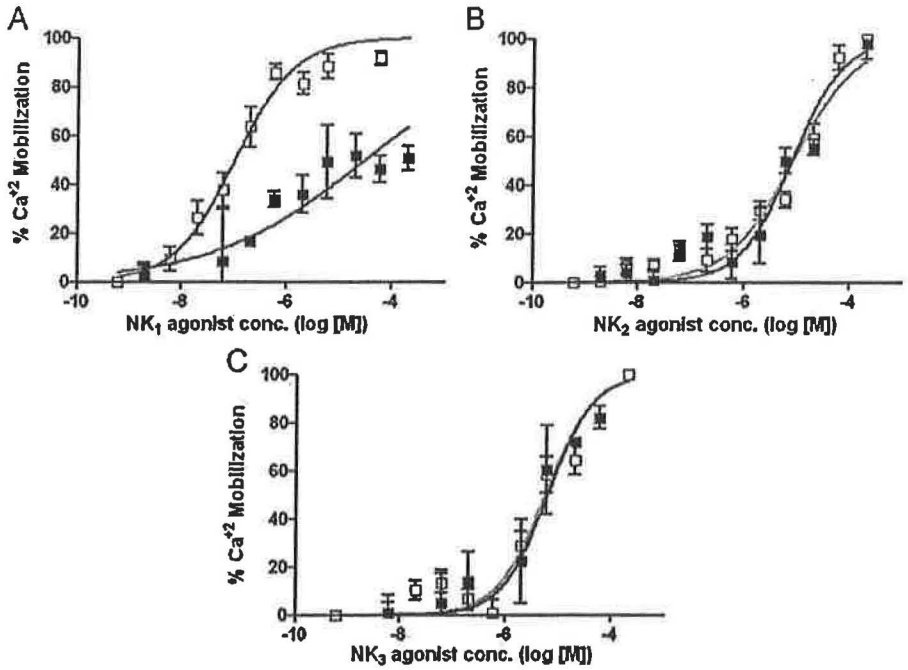

Fig. 3. Effect of palonosetron and netupitant on selective NK_1, NK_2 and NK_3 agonist -induced calcium-ion mobilization in NG108-15 cells. NG108-15 cells were pre-incubated with netupitant (30 nM) and palonosetron (6 nM) for 1 h at 37 °C. Parallel incubations without antagonist were used as controls. After removal of palonosetron and netupitant, cells were incubated at 37 °C for 1 h with various concentrations of selective agonists to NK receptors before measurement of calcium-ion mobilization. (A) Empty squares and gray trace: response to selective NK_1 agonist ([Sar^9, Met $(O_2)^{11}$]—Substance P); $EC_{50}=0.1\pm0.03$ μM ($n=7$). Filled squares and black trace: response to NK_1 agonist plus netupitant and palonosetron; $EC_{50}=30\pm15$ μM ($n=3$). (B) Empty squares and gray trace: response to selective NK_2 agonist (GR 64349); $EC_{50}=8\pm2$ μM ($n=9$). Filled squares and black trace: response to NK_2 agonist plus netupitant and palonosetron; $EC_{50}=8\pm2$ μM ($n=4$). (C) Empty squares and gray trace: response to selective NK_3 agonist (Senktide); $EC_{50}=6\pm1$ μM ($n=9$). Filled squares and black trace: response to NK_3 agonist plus netupitant and palonosetron; $EC_{50}=6\pm4$ μM ($n=3$). Error bars correspond to ±S.E.M.

Palonosetron and netupitant significantly inhibited the selective NK_1 receptor agonist effect on calcium-ion mobilization; the EC_{50} shifted to the right approximately 300-fold from 0.1 ± 0.03 μM when using [Sar^9, Met $(O_2)^{11}$]—Sub-P to 30 ± 15 μM when using [Sar^9, Met $(O_2)^{11}$]—Sub-P plus netupitant and palonosetron (Fig. 3A). In contrast, netupitant plus palonosetron did not inhibit the selective NK_2 or NK_3 receptor agonist-induced calcium-ion mobilization (Fig. 3B and C).

4. Discussion

Recent studies showed that palonosetron inhibits SP mediated responses in vitro and in vivo possibly as a result of inhibition of 5-HT_3/NK_1 receptor crosstalk. Specifically, palonosetron was found to inhibit 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 when using ondansetron or granisetron, two other commonly used 5-HT_3 receptor antagonists (Rojas et al., 2010b). These results helped provide a rationale for palonosetron's unique ability among 5-HT_3 receptor antagonists to prevent nausea and delayed emesis after moderate emetogenic chemotherapy. Given the inhibition of delayed emesis by NK_1 receptor antagonists, it is not clear if palonosetron's inhibition would make any difference if co-administered with an NK_1 receptor antagonist. In order to address this question, we studied the inhibition of SP-induced calcium-ion mobilization in NG108-15 cells by palonosetron and netupitant, a selective NK_1 receptor antagonist.

During preliminary experiments with 5-HT_3 receptor antagonists, preincubation of NG108-15 cells with a saturating concentration of palonosetron in the absence of serotonin significantly inhibited the SP response. In contrast, the SP response was not affected by preincubation of cells with saturating concentrations of either granisetron or ondansetron (Fig. 1). The results showed that we could differentiate palonosetron's inhibition of SP-induced calcium-ion mobilization from its inhibition of serotonin-induced calcium-ion mobilization when using these cells. The results also suggested that palonosetron's ability to trigger 5-HT_3 receptor internalization and subsequently inhibit SP-induced responses can occur even in the absence of serotonin. The mechanism by which palonosetron inhibits NK_1 signaling is unclear. It is possible that palonosetron-induced 5-HT_3 receptor internalization causes a down regulation of second messenger systems which limits NK_1 signaling. Alternatively, the internalization of the 5-HT_3 receptor could lead to subsequent partial internalization of the NK_1 receptor. A case of a ligand binding to one receptor and triggering internalization of another receptor has been reported recently: NMDA binding to the NMDA receptor triggers internalization of the $GABA_B$ receptor. (Guetg et al., 2010). It is noteworthy that palonosetron did not completely eliminate NK_1 receptor signaling (Fig. 1); this is in accord with clinical data showing that palonosetron's antiemetic efficacy can be enhanced when dosed with an NK_1 receptor antagonist (Longo et al., 2010; Schwartzberg et al., 2011). Another possibility is that the receptor–palonosetron complex could be targeted for receptor degradation rather than recycling to the cell surface with a concomitant disturbance of downstream 5-HT_3/NK_1 signaling interactions. Relevant to this possibility, 5-HT_3 receptor replenishment at the cell surface after palonosetron-triggered internalization does not occur within the first 2.5 h of exposure to the antagonist (Rojas et al., 2010a), whereas 5-HT_3 receptors reappear at the surface 1 h after serotonin-induced internalization (Freeman et al., 2006; Rojas et al., 2010a). Second messenger

systems that are activated following NK_1 receptor signaling could also be inhibited after exposure of cells to palonosetron. In addition to calcium-ion mobilization, stimulation of phosphoinositol turnover, arachidonic acid mobilization, and cyclic AMP accumulation have all been associated with the NK_1 receptor response (Quartara and Maggi, 1997). The details of palonosetron's effects on NK_1 receptor trafficking as well as effects on second messenger systems are the subject of current investigations in our laboratory. Regardless of the selective mechanism however, the ability to discriminate palonosetron's inhibition of the SP versus serotonin response provided a springboard to carry out experiments to determine if palonosetron's inhibition of the SP response could alter the effect of NK_1 receptor antagonists.

We proceeded to determine the separate effect of palonosetron and of an NK_1 receptor antagonist (netupitant) on the SP response at various concentrations. We decided to use the combination of palonosetron and netupitant because this $5HT_3$/NK_1 receptor antagonist antiemetic combination is currently in clinical development. Netupitant did not inhibit the SP response at 1 nM; it increasingly inhibited the SP response at 3 and 10 nM and exhibited maximal inhibition at 30 nM (Table 1 and Fig. 2A). Similarly, palonosetron did not inhibit the SP response at 0.2 nM; it showed increasing inhibition at 0.6 and 2 nM and exhibited maximal inhibition of the SP response at 6 nM (Table 1, Fig. 2B).

These results provided the information on the concentrations to use in the subsequent set of experiments when both the antagonists were used in combination. We first determined the effect on SP response when both palonosetron and netupitant were present at threshold concentrations, i.e., concentrations at which neither antagonist inhibited the SP response: netupitant at 1 nM and palonosetron at 0.2 nM. In this case there was an approximately 60-fold shift in EC_{50} of the SP response when compared to the EC_{50} in the presence of each antagonist alone (Table 1, Fig. 2C). We then determined the effect on the SP response when both palonosetron and netupitant were present at concentrations where maximal inhibition was observed with each antagonist: netupitant at 30 nM and palonosetron at 6 nM. Under these conditions, the EC_{50} represented a 22-fold shift to the right with respect to maximal inhibition by netupitant ($P<0.001$) and a 30-fold increase in EC_{50} with respect to maximal inhibition by palonosetron ($P<0.001$) (Table 1, Fig. 2D). The maximum response when using palonosetron plus netupitant was not reached; this is the hallmark of lower receptor availability, a finding consistent with NK_1 receptor internalization. The results showed a synergistic effect on the inhibition of the SP response regardless of whether palonosetron and netupitant were present at threshold or maximal inhibitory concentrations. Synergistic activity between drugs in other systems has been reported previously. For example, addition of the protein tyrosine kinase inhibitor genistein has been shown to synergistically increase the antiproliferative effect of camptothecins in a variety of cell lines (Papazisis et al., 2006). In another study, when combined with inactive doses of 5-fluorouracil (5-FU), the antiangiogenic drug rapamycin significantly improved outcomes in a murine colon adenocarcinoma model as compared to rapamycin dosing alone (Seeliger et al., 2004). Systematic screening for synergistic drug combinations has also been the subject of recent investigations (Borisy et al., 2003; Li et al., 2011).

Receptor cross-talk can be defined as the activation of one receptor by its ligand that in turn influences the cellular responses to another receptor system. Crosstalk between the $5\text{-}HT_3$ and NK_1 receptor signaling systems has been demonstrated by several independent laboratories. For example, $5\text{-}HT_3$ receptor antagonists have been shown to block SP-mediated vagal afferent activation (Minami et al., 2001). In addition, NK_1 antagonism can block serotonin-induced vagal afferent activation (Minami et al., 2001). In separate studies, SP, an agonist at the NK_1 receptor, was shown to potentiate $5\text{-}HT_3$ receptor mediated current in rat trigeminal ganglion neurons (Hu et al., 2004). In another set of studies, palonosetron, an antagonist of the $5\text{-}HT_3$ receptor, was shown to inhibit 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 (Rojas et al., 2010b). Consequently, the potential inhibition of downstream receptor crosstalk, in addition to direct receptor antagonism, represents an additional layer of inhibition that could trigger synergistic inhibitory effects. Recent studies have reported synergistic antiemetic interactions brought about by a combination of tropisetron, a $5\text{-}HT_3$ antagonist and CP 99994 an NK_1 receptor antagonist against vomiting in the least shrew (Darmani et al., 2011). However, synergism against emesis in this case was observed only at a small dose range due to tropisetron's ability to act as partial agonist at higher concentrations.

Given the relevance of the NK_1 receptor to emesis, we wanted to rule out the possibility that the SP effects on calcium-ion mobilization and the corresponding inhibition by palonosetron and netupitant observed in these cells were not occurring through other NK receptors. Experiments with selective NK_1 and NK_2 selective receptor agonists exhibited calcium-ion mobilization in a dose-response manner indicating that these three receptors are found in NG108-15 cells. Consequently, it was possible that these receptors and their signaling could be at least partially responsible for the observed inhibition. In order to rule out this possibility, we looked to see if palonosetron and netupitant could inhibit the effect of these selective NK receptor agonists. Palonosetron plus netupitant significantly inhibited the selective NK_1 receptor agonist effect on calcium-ion mobilization; EC_{50} shifted to the right approximately 300-fold (Fig. 3A). In contrast, palonosetron plus netupitant did not inhibit the selective NK_2 or NK_3 receptor agonist-induced calcium-ion mobilization (Fig. 3B and C). The results indicated that inhibition of calcium-ion mobilization by palonosetron and netupitant in these cells must be occurring through the NK_1 receptor.

It is important to point out that these studies use NG108-15 cells as a model of the potential mechanism of action on delayed emesis when using palonosetron and netupitant in humans. Clearly, NG108-15 cells do not incorporate the complexities of a whole organism and the results obtained in these studies are only a first approximation of what could be observed in the clinic.

In summary, the results suggest that not all $5\text{-}HT_3$ receptor antagonists are the same. Palonosetron, but not ondansetron or granisetron could inhibit the SP response in NG108-15 cells in the absence of serotonin. Further, inhibition by palonosetron and NK_1 antagonists of the SP response in NG108-15 cells is synergistic. This inhibition is selective to the NK_1 receptor and its crosstalk with the $5\text{-}HT_3$ receptor signaling system. $5\text{-}HT_3$ antagonists that inhibit $5\text{-}HT_3$/NK_1 receptor signaling crosstalk in addition to antagonizing the $5\text{-}HT_3$ receptor could bring about an enhanced antiemetic effect during nausea and delayed emesis. The results support the use of the combination of palonosetron and NK-1 receptor antagonists in the prevention of chemotherapy induced nausea and vomiting.

References

Aapro, M.S., Grunberg, S.M., Manikhas, G.M., Olivares, G., Suarez, T., Tjulandin, S.A., Bertoli, L.F., Yunus, F., Morrica, B., Lordick, F., Macciocchi, A., 2006. A phase III, double-blind, randomized trial of palonosetron compared with ondansetron in preventing chemotherapy-induced nausea and vomiting following highly emetogenic chemotherapy. Ann. Oncol. 17, 1441–1449.

Basch, E., Prestrud, A.A., Hesketh, P.J., Kris, M.G., Feyer, P.C., Somerfield, M.R., Chesney, M., Clark-Snow, R.A., Flaherty, A.M., Freundlich, B., Morrow, G., Rao, K.V., Schwartz, R.N., Lyman, G.H., 2011. Antiemetics: american society of

clinical oncology clinical practice guideline update. J. Clin. Oncol. 29, 4189–4198.

Borisy, A.A., Elliott, P.J., Hurst, N.W., Lee, M.S., Lehar, J., Price, E.R., Serbedzija, G., Zimmermann, G.R., Foley, M.A., Stockwell, B.R., Keith, C.T., 2003. Systematic discovery of multicomponent therapeutics. Proc. Natl. Acad. Sci. USA 100, 7977–7982.

Darmani, N.A., Ray, A.P., 2009. Evidence for a re-evaluation of the neurochemical and anatomical bases of chemotherapy-induced vomiting. Chem. Rev. 109, 3158–3199.

Darmani, N.A., Chebolu, S., Amos, B., Alkam, T., 2011. Synergistic antiemetic interactions between serotonergic 5-HT(3) and tachykininergic NK(1)-receptor antagonists in the least shrew (Cryptotis parva). Pharmacol. Biochem. Behav. 99, 573–579.

Eisenberg, P., Figueroa-Vadillo, J., Zamora, R., Charu, V., Hajdenberg, J., Cartmell, A., Macciocchi, A., Grunberg, S., 2003. Improved prevention of moderately emetogenic chemotherapy-induced nausea and vomiting with palonosetron, a pharmacologically novel 5-HT3 receptor antagonist: results of a phase III, single-dose trial versus dolasetron. Cancer 98, 2473–2482.

Emerit, M.B., Riad, M., Fattaccini, C.M., Hamon, M., 1993. Characteristics of [14C] guanidinium accumulation in NG 108–15 cells exposed to serotonin 5-HT3 receptor ligands and substance P. J. Neurochem. 60, 2059–2067.

Ettinger, D.S., Armstrong, D.K., Barbour, S., Berger, M.J.P.J.B., Bradbury, B., Ellis, G., Kirkegaard, S., Kloth, D.D., Kris, M.G., Lim, D., Boehnke Michaud, L., Nabati, L., Noonan, K., Rugo, H.S., Siler, D., Sorscher, S.M., Stelts, S., Stucky-Marshall, L., Todaro, B., Urba, S., 2011. NCCN Clinical Practice Guidelines in Oncology (NCCN GuidelinesTM) Antiemesis, version 1.2012. NCCN.org.

Feyer, P., Jordan, K., 2011. Update and new trends in antiemetic therapy: the continuing need for novel therapies. Ann. Oncol. 22, 30–38.

Freeman, S.L., Glatzle, J., Robin, C.S., Valdellon, M., Sternini, C., Sharp, J.W., Raybould, H.E., 2006. Ligand-induced 5-HT3 receptor internalization in enteric neurons in rat ileum. Gastroenterology 131, 97–107.

Gralla, R., Lichinitser, M., Van Der Vegt, S., Sleeboom, H., Mezger, J., Peschel, C., Tonini, G., Labianca, R., Macciocchi, A., Aapro, M., 2003. Palonosetron improves prevention of chemotherapy-induced nausea and vomiting following moderately emetogenic chemotherapy: results of a double-blind randomized phase III trial comparing single doses of palonosetron with ondansetron. Ann. Oncol. 14, 1570–1577.

Guetg, N., Abdel Aziz, S., Holbro, N., Turecek, R., Rose, T., Seddik, R., Gassmann, M., Moes, S., Jenoe, P., Oertner, T.G., Casanova, E., Bettler, B., 2010. NMDA receptor-dependent GABAB receptor internalization via CaMKII phosphorylation of serine 867 in GABAB1. Proc. Natl. Acad. Sci. USA 107, 13924–13929.

Hesketh, P.J., Van Belle, S., Aapro, M., Tattersall, F.D., Naylor, R.J., Hargreaves, R., Carides, A.D., Evans, J.K., Horgan, K.J., 2003. Differential involvement of neurotransmitters through the time course of cisplatin-induced emesis as revealed by therapy with specific receptor antagonists. Eur. J. Cancer 39, 1074–1080.

Hu, W.P., You, X.H., Guan, B.C., Ru, L.Q., Chen, J.G., Li, Z.W., 2004. Substance P potentiates 5-HT3 receptor-mediated current in rat trigeminal ganglion neurons. Neurosci. Lett. 365, 147–152.

Li, S., Zhang, B., Zhang, N., 2011. Network target for screening synergistic drug combinations with application to traditional Chinese medicine. BMC Syst. Biol. 5 (Suppl 1), S10.

Longo, F., Mansueto, G., Lapadula, V., De Sanctis, R., Quadrini, S., Grande, R., Gori, B., Altavilla, A., D'Antoni, I., Del Signore, E., Stumbo, L., De Luca, C., Cimadon, B., Cortesi, E., Gamucci, T., Di Seri, M., 2010. Palonosetron plus 3-day aprepitant and dexamethasone to prevent nausea and vomiting in patients receiving highly emetogenic chemotherapy. Support. Care Cancer 19, 1159–1164.

Minami, M., Endo, T., Yokota, H., Ogawa, T., Nemoto, M., Hamaue, N., Hirafuji, M., Yoshioka, M., Nagahisa, A., Andrews, P.L., 2001. Effects of CP-99, 994, a tachykinin NK(1) receptor antagonist, on abdominal afferent vagal activity in ferrets: evidence for involvement of NK(1) and 5-HT(3) receptors. Eur. J. Pharmacol. 428, 215–220.

Oo, T.H., Hesketh, P.J., 2005. Further improvement in the control of chemotherapy-induced nausea and vomiting: future directions. In: Hesketh, P.J. (Ed.), Management of Nausea and Vomiting in Cancer and Cancer Treatment. Jones and Bartlett Publishers, Inc, London, pp. 231–245.

Papazisis, K.T., Kalemi, T.G., Zambouli, D., Geromichalos, G.D., Lambropoulos, A.F., Kotsis, A., Boutis, L.L., Kortsaris, A.H., 2006. Synergistic effects of protein tyrosine kinase inhibitor genistein with camptothecins against three cell lines in vitro. Cancer Lett. 233, 255–264.

Quartara, L., Maggi, C.A., 1997. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides 31, 537–563.

Reiser, G., Hamprecht, B., 1989. Substance P and serotonin act synergistically to activate a cation permeability in a neuronal cell line. Brain Res. 479, 40–48.

Roila, F., Herrstedt, J., Aapro, M., Gralla, R.J., Einhorn, L.H., Ballatori, E., Bria, E., Clark-Snow, R.A., Espersen, B.T., Feyer, P., Grunberg, S.M., Hesketh, P.J., Jordan, K., Kris, M.G., Maranzano, E., Molassiotis, A., Morrow, G., Olver, I., Rapoport, B.L., Rittenberg, C., Saito, M., Tonato, M., Warr, D., 2010. Guideline update for MASCC and ESMO in the prevention of chemotherapy- and radiotherapy-induced nausea and vomiting: results of the Perugia consensus conference. Ann. Oncol. 21 (Suppl 5) v232–243.

Rojas, C., Thomas, A.G., Alt, J., Stathis, M., Zhang, J., Rubenstein, E.B., Sebastiani, S., Cantoreggi, S., Slusher, B.S., 2010a. Palonosetron triggers 5-HT(3) receptor internalization and causes prolonged inhibition of receptor function. Eur. J. Pharmacol. 626, 193–199.

Rojas, C., Li, Y., Zhang, J., Stathis, M., Alt, J., Thomas, A.G., Cantoreggi, S., Sebastiani, S., Pietra, C., Slusher, B.S., 2010b. The antiemetic 5-HT3 receptor antagonist Palonosetron inhibits substance P-mediated responses in vitro and in vivo. J. Pharmacol. Exp. Ther. 335, 362–368.

Saito, M., Aogi, K., Sekine, I., Yoshizawa, H., Yanagita, Y., Sakai, H., Inoue, K., Kitagawa, C., Ogura, T., Mitsuhashi, S., 2009. Palonosetron plus dexamethasone versus granisetron plus dexamethasone for prevention of nausea and vomiting during chemotherapy: a double-blind, double-dummy, randomised, comparative phase III trial. Lancet Oncol. 10, 115–124.

Schwartzberg, L., Jackson, J., Jain, G., Balu, S., Buchner, D., 2011. Impact of 5-HT(3) RA selection within triple antiemetic regimens on uncontrolled highly emetogenic chemotherapy-induced nausea/vomiting. Expert. Rev. Pharmacoecon. Outcomes Res. 11, 481–488.

Seeliger, H., Guba, M., Koehl, G.E., Doenecke, A., Steinbauer, M., Bruns, C.J., Wagner, C., Frank, E., Jauch, K.W., Geissler, E.K., 2004. Blockage of 2-deoxy-D-ribose-induced angiogenesis with rapamycin counteracts a thymidine phosphorylase-based escape mechanism available for colon cancer under 5-fluorouracil therapy. Clin. Cancer Res. 10, 1843–1852.