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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.

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 pre-incubated 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

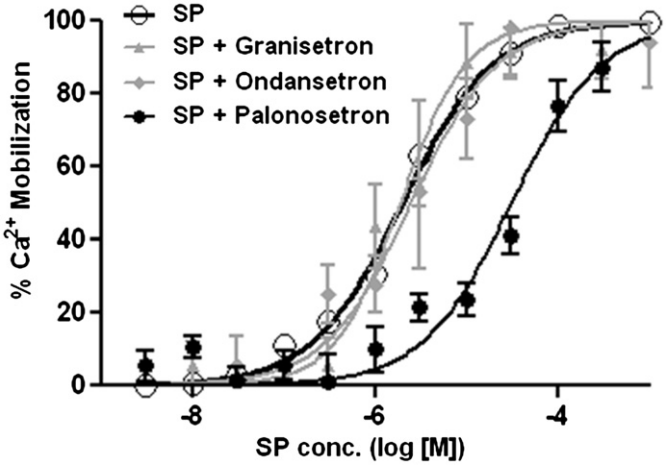

Fig. 1. Effect of 5-HT_3 receptor antagonists on SP induced calcium-ion mobilization in NG108-15 cells. Cells were pre-incubated with saturating concentrations (≥30-fold K_d) of granisetron (50 nM), ondansetron (300 nM) or palonosetron (10 nM) for 1 h at 37 °C. Subsequent to complete removal of antagonists, 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.