

In vitro and in vivo pharmacological characterization of Pronetupitant, a prodrug of the neurokinin 1 receptor antagonist Netupitant



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ABSTRACT

The aim of the present study was to investigate the pharmacological activity of Pronetupitant, a novel compound designed to act as prodrug of the NK₁ antagonist Netupitant. In receptor binding experiments Pronetupitant displayed high selectivity for the NK₁ receptor. In a calcium mobilization assay performed on CHO_{NK1} cells Pronetupitant (100 nM, 15 min preincubation) behaved as an NK₁ antagonist more potent than Netupitant (pK_B 8.72 and 7.54, respectively). In the guinea pig ileum bioassay Pronetupitant antagonized the contractile effect of SP showing a similar potency as Netupitant ($pK_B \approx 9$). Similar results were obtained with 5 min preincubation time while at 2 min only Pronetupitant produced significant effects. In vivo in mice the intrathecal injection of 0.1 nmol SP elicited the typical scratching, biting and licking (SBL) nociceptive response. This effect of SP was dose dependently (0.1–10 mg/kg) antagonized by Pronetupitant given intravenously 2 h before the peptide. Superimposable results were obtained using Netupitant. Pharmacokinetic studies performed in rats demonstrate that Pronetupitant, after i.v. administration, is quickly (few minutes) and completely converted to Netupitant. Collectively the present results indicated that Pronetupitant acts in vitro as selective NK₁ antagonist more potent than Netupitant. However based on the short half-life measured for Pronetupitant in rats, the in vivo action of Pronetupitant can be entirely interpreted as due to its conversion to Netupitant.

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Introduction

Substance P (SP) is an undecapeptide that acts as neuromodulator by binding and activating a G protein coupled receptor named NK₁ receptor. SP and its receptor are widely distributed in the central and peripheral nervous system where they modulate several biological functions, such as pain and migraine, inflammatory conditions, mood and anxiety, drug abuse, nausea and vomiting [1]. The NK₁ antagonist Aprepitant and its prodrug Fosaprepitant have been approved to treat chemotherapy-induced nausea and vomiting (CINV), and are commonly used in association with 5-HT₃ receptor antagonists and dexamethasone [6]. Netupitant (Fig. 1) is a potent, selective and orally active NK₁ receptor antagonist [12,19]. This

compound, in combination with the 5-HT₃ antagonist Palonosetron (Aloxi), has been recently approved by FDA (brand name Akynzeo®) for the treatment of CINV [2,8,11]. Of note, Netupitant is poorly soluble in water and this prevents its formulation in a vehicle suitable for intravenous administration in humans. Pronetupitant (proposed INN name Fosnetupitant) is a methylene phosphate prodrug of Netupitant (Fig. 1) designed to overcome this limitation. Thus, Pronetupitant can represent an intravenous alternative to the oral Netupitant. The aim of the present study was to characterize the pharmacological activity of Pronetupitant in vitro and in vivo. In vitro Pronetupitant has been evaluated in receptor binding studies, in the calcium mobilization assay performed in CHO cells stably transfected with the human NK₁ receptor (CHO_{NK1}), and in the guinea pig ileum (gpi) bioassay. The activity of Pronetupitant was compared to that of Netupitant and of the standard NK₁ antagonists Aprepitant and Fosaprepitant. The pharmacokinetic properties of Pronetupitant, compared to those of Netupitant, were evaluated in the rat. Finally, Netupitant and Pronetupitant were assessed in vivo in mice in the scratching, biting and licking (SBL) test.

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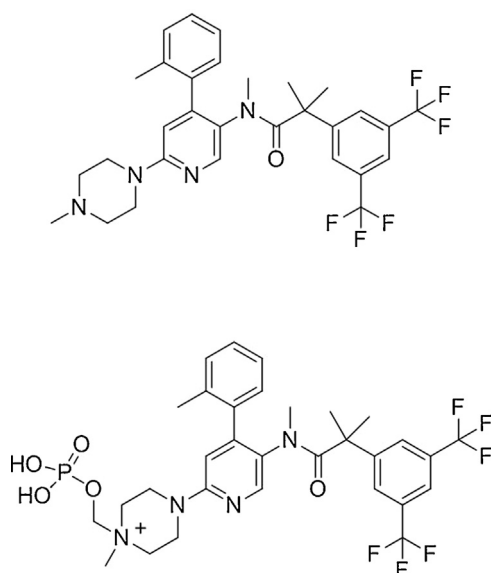


Fig. 1. Chemical structure of Netupitant (top panel) and Pronetupitant (bottom panel).

Methods

Receptor binding screening profile

Pronetupitant was assessed in receptor binding experiments performed on membranes of CHO cells expressing the 3 human tachykinins receptors (NK_1 , NK_2 and NK_3) as well as 50 different GPCR, monoamine transporters and ion channels. This study was performed under contract with the service company CEREP SA (Celle l'Evescault, France).

Calcium mobilization assay

CHO $_{NK1}$ cells were a generous gift from the laboratories of Prof. T. Costa (ISS, Rome, IT). CHO $_{NK1}$ cells were maintained in DEMEM/F-12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, geneticin (0.2 mg/l), and cultured at 37 °C in 5% CO₂ humidified air. CHO $_{NK1}$ cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates. The following day, the cells were incubated with medium supplemented with 2.5 mM probenecid, 3 μ M of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. After that time the loading solution was aspirated and 100 μ l/well of assay buffer (Hank's Balanced Salt Solution, HBSS) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2.5 mM probenecid and 500 μ M Brilliant Black (Sigma-Aldrich, St. Louis, MO, USA) was added. Concentrated solution (1 mM) of SP was made in bidistilled water and kept at –20 °C. Netupitant, Pronetupitant, Aprepitant and Fosaprepitant were solubilized in dimethylsulfoxide (DMSO) at a final concentration of 10 mM and stock solutions were kept at –20 °C until use. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% bovine serum albumin fraction V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured. On-line additions were carried out in a volume of 50 μ l/well. To facilitate drug diffusion into the wells, the present studies were performed at 37 °C and three cycles of mixing (25 μ l from each well moved up and down 3 times) were performed immediately after antagonist injection to the wells. Netupitant, Pronetupitant, Aprepitant and Fosaprepitant were injected into the

wells 2, 5 and 24 min before adding SP. Maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response.

Animals experiments

All the study involving animals complied with the standard of the European Communities Council directives (86/609/EEC) and national regulations (D. L. 116/92). This research was reported following the ARRIVE guidelines [15]. Male guinea pigs (300–350 g, Harlan, Udine, Italy) and male CD-1 mice (25–28 g, Harlan, Udine, Italy) were used in a number of 6 and 143, respectively. Guinea pigs were housed in 300 mm $\times$ 570 mm $\times$ 155 mm cages (Tecniplast, Buguggiate, VA, Italy), 5 guinea pigs/cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am), with food (standard diet, Mucedola, Settimo Milanese, Italy) and water ad libitum for at least 7 days before the experiment. Mice were housed in 267 mm $\times$ 207 mm $\times$ 140 mm cages (Tecniplast, Buguggiate, VA, Italy), 5 mice/cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am) with food (standard diet, Mucedola, Settimo Milanese, Italy) and water ad libitum for at least 7 days before experiments began. Each animal was used only once. Each cage was provided with a mouse red house (Tecniplast, Buguggiate, VA, Italy) and nesting materials. For the pharmacokinetic studies Wistar Hannover male rats (225–275 g, Harlan, Udine, Italy) were used in a total number of 60. Rats were housed in 595 mm $\times$ 385 mm $\times$ 200 mm cages (Tecniplast, Buguggiate, VA, Italy), up to 5 to a cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am), with food (standard diet, Mucedola, Settimo Milanese, Italy) and water ad libitum for at least 15 days before the experiment. Animals were randomly assigned to the experimental groups.

Bioassay studies in the guinea pig ileum

After killing the animal via isoflurane overdose, ileum segments were taken and suspended in 5 ml organ baths containing Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 1.8, glucose 10, hexamethonium bromide 2.2 μ M and benadril 0.3 μ M) oxygenated with 95% O₂ and 5% CO₂. The temperature was set at 37 °C. A resting tension of 1 g was applied to the tissues. Tissue contractions were measured isototically through strain gauge transducers (Ugo Basile, Gemonio, VA, Italy) and recorded with a PC-based acquisition system (Power Lab, 4/25, ADInstruments, Dunedin, New Zealand). After an equilibration period of about 60 min, to check the responsiveness of the preparation, the contractile effect of carbachol 10 μ M was recorded. After approximately 20 min, atropine 1 μ M was added to prevent NK₃ induced acetylcholine release and 20 min later a cumulative concentration–response curve to SP was performed (0.1–100 nM). In the same tissues, a second concentration response curve to SP was tested in presence of antagonists (10 nM) with 15 min pre-incubation time. In some tissues the repeatability of the concentration response curve to SP has been assessed. In a separate series of experiments the compounds under study were challenged vs the contractive action of SP 10 nM at different incubation times (0, 2, and 5 min). Substance P, atropine and carbachol were solubilized in saline at the concentration of 1 mM, whereas the antagonists were suspended in DMSO at the concentration of 10 mM; the successive dilutions were made in saline. Stock solutions were kept at –20 °C until use.

Scratching, biting and licking (SBL) test

SP 0.1 nmol was given intrathecally (i.t.). I.t. injections (5 μ l per mouse) were given under light (just sufficient to produce a loss of

the righting reflex) isoflurane anesthesia according to the procedure described by Hylden and Wilcox [14] and routinely adopted in our laboratory [20]. Approximately 30–45 min before i.t. injection, the mice were adapted to an individual plastic cage which served as observation chamber. Immediately after the injection of SP mice were individually observed for 5 min. The total time (s) spent by the animal displaying the following behaviors was recorded: hind limb scratching directed toward the flank, biting or licking of the fore and hind paw, and biting or licking of the tail [21,24]. Netupitant and Pronetupitant (0.1, 1 and 10 mg/kg, i.v.) were administered 120 min before SP. All experiments were started at 9.00 a.m. SP was solubilized in saline. Netupitant was dissolved in water containing 5% DMSO and 5% Tween 80 (Sigma–Aldrich, St. Louis, MO, USA), Pronetupitant was solubilized in water. Vehicle solution (5% DMSO and Tween 80 5% in water) was used as control.

Pharmacokinetic studies

Netupitant (10 mg/kg, 0.4% solfobutyl ether beta-cyclodextrin, 5% glucose in water) and Pronetupitant (13.16 mg/kg, 5% glucose, equimolar doses of Netupitant and Pronetupitant have been selected for this study) were administered by intravenous bolus injection into the rat tail vein over a period of approximately 3 ml/min and in a dose volume of 5 ml/kg. All animals were dosed once. Blood samples were collected at the following 11 time points: pre-dose, immediately after dosing, 5, 15, 30 min and 1, 2, 4, 8, 24 and 48 h starting from the end of administration. At each sampling time, approximately 0.4 mL blood samples were collected under light isoflurane anesthesia from the retro-orbital sinus of 3 rats per sampling time, each animal being sampled on each day at a maximum of 2–3 alternating time points. Samples were transferred into tubes containing EDTA/NaF anticoagulant, centrifuged at room temperature and the plasma divided into two aliquots and frozen at -20°C . For brain collection, rats were killed with carbon dioxide at each of the following 6 time points: 5, 15, 30 min and 2, 8 and 24 h after the end of the intravenous administration. Brain was collected, immediately frozen in liquid nitrogen and stored at -80°C until analysis. The plasma and brain samples were assayed to determine the concentration of Pronetupitant and Netupitant, according to a validated HPLC-MS/MS method. The following pharmacokinetic parameters were reported: C_{max} , maximum observed plasma concentration (ng/ml for plasma and ng/g for brain); t_{max} , time to reach maximum plasma concentration (h); $t_{1/2}$, elimination half-life (h); AUC, area under the concentration–time curve calculated by the linear trapezoidal rule ($\text{ng} \times \text{h/ml}$); AUC ratio, AUC Pronetupitant/AUC Netupitant (%), CL, clearance. In rats injected with Pronetupitant the concentrations of both Pronetupitant and Netupitant were measured. The AUC ratio Pronetupitant–Netupitant has been assessed in these rats.

Drugs and reagents

SP, atropine and carbachol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Netupitant, Pronetupitant, Aprepitant and Fosaprepitant were from Helsinn Laboratories (Lugano, CH, Switzerland).

Data analysis and terminology

All in vitro data are expressed as means $\pm$ standard error (SEM) of n experiments. For potency values 95% confidence limits were indicated. Agonist potencies are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. Concentration response curve to agonists were fitted with the following equation:

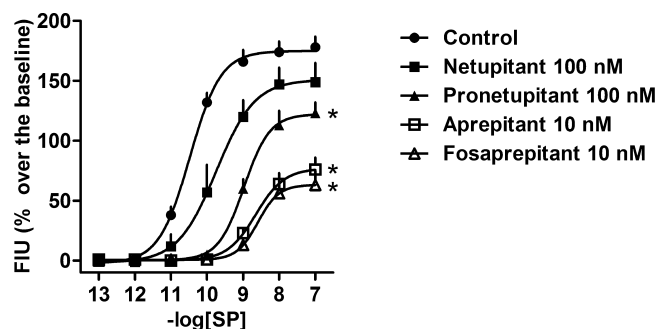


Fig. 2. Calcium mobilization assay. Concentration response curve to SP obtained in the absence (control) and in presence of Netupitant, Pronetupitant, Aprepitant and Fosaprepitant, injected 24 min before SP. Data are mean $\pm$ SEM of 3 separate experiments made in duplicate, * $p < 0.05$ vs control, according to one-way ANOVA followed by the Dunnett's post hoc test. FIU, fluorescence intensity units.

$$\text{Effect} = \text{baseline} + (E_{\text{max}} - \text{baseline}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$$

where X is the agonist concentration.

Antagonist potency was expressed as pK_B calculated as described by Gaddum et al. (1955) [7]. In practice, equieffective concentrations of the agonist in the absence and presence of a noncompetitive antagonist ($[A]$) are compared in a double reciprocal plot describing a straight line, and pK_B was derived from the equation:

$$\text{pK}_B = \log_{10}((\text{slope} - 1) / [A])$$

All in vivo data are expressed as mean $\pm$ SEM of n animals (3–4 separate experiments). Data have been statistically analyzed with one way ANOVA followed by the Dunnett's test; p values less than 0.05 were considered statistically significant. For all the non-linear regression analysis of the in vitro data and the statistical analyses the GraphPad Prism software (v.5.0; GraphPad, La Jolla, CA, USA) was used. All the pharmacokinetic parameters were calculated by the Kinetica™, version 4.4.1, PK/PD Analysis software (Thermo Electron Corporation Informatics, Philadelphia, USA)

Results

Receptor binding screening profile

Pronetupitant bound the human NK_1 receptor with sub-nanomolar affinity ($\text{pK}_i \sim 9.5$). Pronetupitant displayed micromolar ($\text{pK}_i \sim 6.1$) affinity for the NK_3 receptor and did not bind to the NK_2 receptor. The selectivity of Pronetupitant has been further investigated at over 50 G-protein coupled receptors and ion channels. The compound displayed micromolar affinity for the 5-HT₆ ($\text{pK}_i \sim 5.2$) receptor and type L Ca^{2+} channels ($\text{pK}_i \sim 5.7$) and did not bind all the other proteins investigated. Thus, in this screening assay Pronetupitant showed at least 1000-fold selectivity for the NK_1 receptor.

Calcium mobilization assay

In the calcium mobilization assay performed on CHO_{NK1} cells, SP increased the intracellular calcium concentrations in a concentration-dependent manner with pEC_{50} and E_{max} values of 10.48 (10.25–10.71) and $175 \pm 9\%$ over the basal values, respectively (Fig. 2). Netupitant (100 nM, 24 min incubation) shifted to right the concentration response curve to SP and produced a statistically significant reduction of agonist maximal effect. Pronetupitant tested at the same concentration and with the same preincubation time mimicked the effect of Netupitant, being

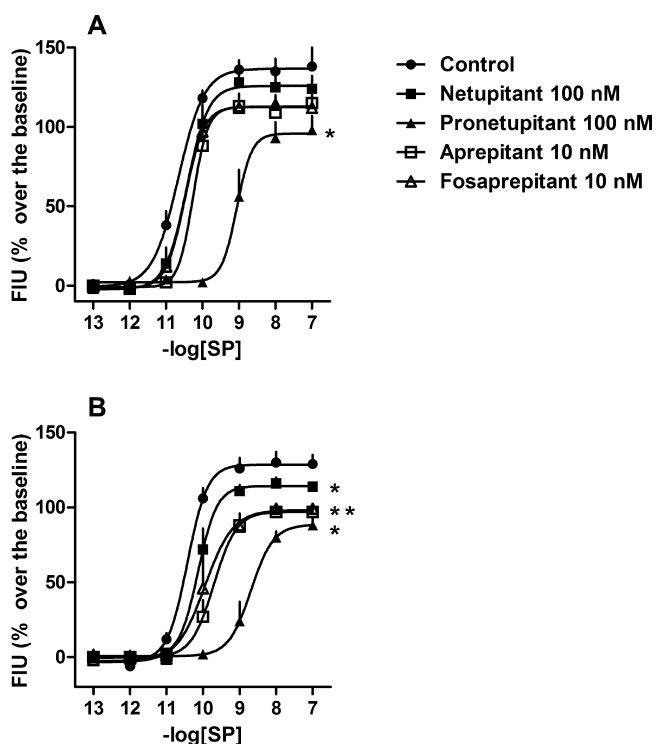


Fig. 3. Calcium mobilization assay. Concentration response curve to SP obtained in the absence (control) and in presence of Netupitant, Pronetupitant, Aprepitant and Fosaprepitant incubated at 2 (panel A) and 5 min (panel B). Data are mean $\pm$ SEM of 3 separate experiments made in duplicate. * $p < 0.05$ vs control, according to one-way ANOVA followed by the Dunnett's post hoc test. FIU, fluorescence intensity units.

~ 10 -folds more potent; pK_B values of 7.54 (6.79–8.29) and 8.72 (8.18–9.26) were extrapolated for Netupitant and Pronetupitant, respectively. Under the same experimental conditions, 10 nM Aprepitant or Fosaprepitant caused a robust rightward shift of the concentration response curve to SP and a strong depression of its E_{max} ; pK_B values of 10.16 (10.00–10.32) and 10.40 (9.67–11.13) were calculated for Aprepitant and Fosaprepitant, respectively (Fig. 2). A separate series of experiments was performed in order to evaluate the compounds effects at different times of incubation (2 and 5 min). In this series of experiments, SP increased the intracellular calcium concentrations in a concentration-dependent manner with pEC_{50} and E_{max} values of 10.66 (10.49–10.83) and $137 \pm 12\%$ over the basal values, respectively (Fig. 3). When the antagonists were incubated for 2 min, the concentration response curve to SP was only poorly affected by Aprepitant, Fosaprepitant and Netupitant. On the contrary a robust inhibitory effect was exerted by Pronetupitant (pK_B 8.85 (8.37–8.33), Fig. 3, panel A). Finally when the incubation time was prolonged for 5 min (Fig. 3, panel B), all compounds elicited a statistically significant antagonist effect against SP induced calcium mobilization. pK_B values of 7.00 (6.37–7.63) and 8.69 (7.29–9.09) were extrapolated for Netupitant and Pronetupitant, respectively. A pK_B of 8.02 (7.32–8.72) was calculated for Aprepitant and Fosaprepitant.

Bioassay studies in the guinea pig ileum

Under the present experimental conditions carbachol 10 μ M elicits a robust contraction of the gpl. The contractile effect of the muscarinic agonist was superimposable at the beginning and at the end of the experiment. As shown in Fig. 4, SP evoked a concentration dependent contractile effect with a pEC_{50} value of 8.27 (8.06–8.48) and maximal effects corresponding to $94 \pm 2\%$ of the contraction elicited by carbachol. Netupitant (10 nM, 15 min of

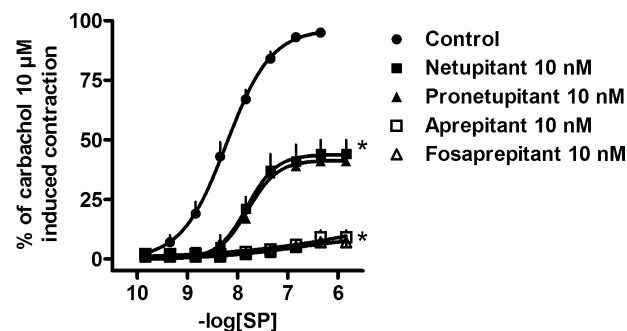


Fig. 4. gpl bioassay. Concentration-response curve to SP obtained in the absence (control) and in presence of Netupitant, Pronetupitant, Aprepitant and Fosaprepitant. Data are mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$ vs control, according to one-way ANOVA followed by the Dunnett's post hoc test. FIU, fluorescence intensity units.

incubation) caused a rightward shift of the concentration response curve to SP associated with a statistically significant depression of the maximal effect elicited by the peptide. Pronetupitant tested at the same concentration and with the same preincubation time mimicked the effect of Netupitant. A pK_B of 8.90 was calculated for Netupitant and Pronetupitant. Under the same experimental conditions 10 nM Aprepitant or Fosaprepitant completely abolished the contraction induced by SP (Fig. 4). A separate series of experiments was performed in order to evaluate the effects of the compounds at different times of incubation (0, 2 and 5 min). In this series of experiments a concentration of Netupitant and Pronetupitant of 100 nM has been used, to compare the action of equi-effective concentrations of Aprepitant and Fosaprepitant and of Netupitant and Pronetupitant. As shown in Fig. 5, SP 10 nM elicited a robust contractile effect corresponding to 70–80% of the contraction elicited by carbachol. None of the NK_1 antagonists investigated in this study was able to counteract SP induced contraction when coinjected with SP (Fig. 5, panel A). When the antagonists were incubated for 2 min, the SP effect was not significantly affected by Aprepitant, Fosaprepitant and Netupitant. On the contrary Pronetupitant almost abolished the contractile effect of SP (Fig. 5, panel B). Finally, when the incubation time was prolonged for 5 min, all compounds significantly counteracted SP induced contractions (Fig. 5, panel C). Of note, all the NK_1 antagonists tested did not modify per se the gpl contraction evoked by carbachol (data not shown).

SBL test

SP (0.1 nmol, i.t.) produced in mice a nociceptive response consisting of hindlimb scratching directed toward the flank, biting and licking of the fore and hind paw, and biting and licking of the tail with no sign of hind limb flaccidity. At the highest dose tested (10 mg/kg, i.v.) both Netupitant and Pronetupitant did not induce, per se, any nociceptive behavior. Moreover no changes in animal gross behavior were observed in mice treated with the two compounds (data not shown). Netupitant (0.1–10 mg/kg, i.v.), given 2 h before SP, was able to significantly counteract, in a dose dependent manner, the nociceptive response induced by SP, inducing statistically significant effects from the dose of 1 mg/kg. Comparable results were obtained when Pronetupitant (0.1–10 mg/kg, i.v.) was injected 2 h before SP (Fig. 6).

Pharmacokinetic studies

Pharmacokinetic studies demonstrated that, after i.v. injection in rats, Pronetupitant was rapidly and extensively converted to Netupitant. In fact, rats treated with Pronetupitant (13.16 mg/kg, i.v.) showed in plasma the C_{max} of prodrug (9,662 ng/ml) soon after

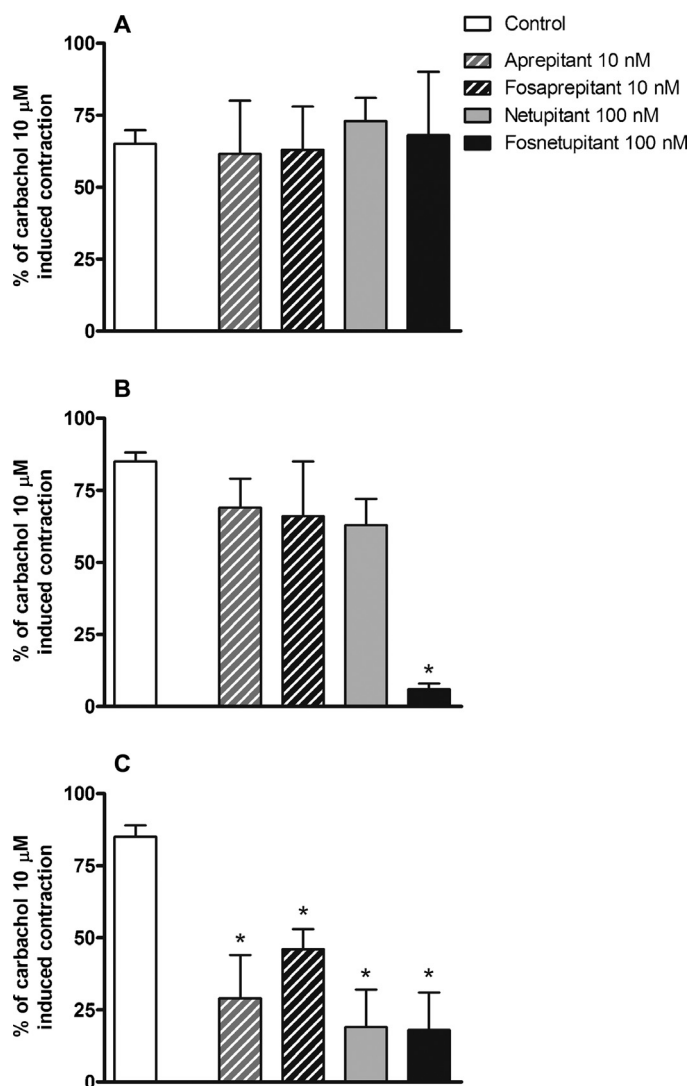


Fig. 5. gpl bioassay. Effects of SP 10 nM obtained in the absence (control) and in presence of Netupitant, Pronetupitant, Aprepitant and Fosaprepitant incubated at different times: 0 (panel A), 2 (panel B) and 5 (panel C) minutes. Data are mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$ vs control, according to one-way ANOVA followed by the Dunnett's post hoc test.

dosing ($t_{\max} \sim 1$ min) and a very low value of half-life ($t_{1/2} = 6$ min). In these rats Netupitant reached the plasma $C_{\max}$ (3,077 ng/ml) ~ 5 min after the injection, and showed a $t_{1/2}$ of 26 h. An AUC ratio Pronetupitant/Netupitant of 1.5% was calculated from these experiments. In the brain Pronetupitant reached a $C_{\max}$ of 4.33 ng/g 15 min from the end of dosing ($C_{\max}$ ratio brain/plasma 0.045%). In the same rats Netupitant reached a brain $C_{\max}$ of 1,335 ng/g 2 h after the injection of Pronetupitant and showed a brain $t_{1/2}$ of 11.5 h. The brain AUC ratio Pronetupitant/Netupitant was 0.01% (Table 1).

In animals injected with Netupitant (10 mg/kg, i.v.), Netupitant reached the plasma $C_{\max}$ (3945 ng/ml) 15 min after the administration and displayed a $t_{1/2}$ of 49 h. In the brain, Netupitant reached a $C_{\max}$ of 12,720 ng/g 5 min after its injection and showed a $t_{1/2}$ of 5.2 h. The Netupitant AUC ratio in plasma was 0.79 comparing the group of rats treated with Pronetupitant and the rats treated with Netupitant.

Discussion and conclusions

Netupitant is a potent and selective orally active NK₁ receptor antagonist [12]. The NK₁ antagonist properties of Netupitant have

been demonstrated in vitro and in vivo. In vitro Netupitant inhibited the increase of intracellular calcium evoked by SP both in CHO_{NK1} [19] and in NG108-15 cells [22] and blocked SP induced contractions of the gpl [19]. Moreover, Netupitant blocked the excitatory effects induced by the selective NK₁ agonist [Sar⁹,Met(O₂)¹¹]SP on dorsal root ganglion neurons [27]. In vivo, the NK₁ antagonist activity of Netupitant has been demonstrated in the mouse SBL and in the gerbil foot tapping assays [19]. Finally, recent preclinical studies demonstrated that Netupitant is able to reduce the symptoms of colitis in the mouse [23] and decreases somatic and visceral neuropathic pain in rats [9]. The combination of Netupitant and Palonosetron (Akynzeo) has been recently approved in US [2,8,11]. Pronetupitant has been designed as a methylene phosphate prodrug of Netupitant, more water soluble than the parent drug. This feature makes Pronetupitant suitable for the intravenous administration in human. Of note, the availability of an intravenous formulation is important for all those patients unable to tolerate oral administration during an episode of nausea or vomiting or as more easy to administer in the concomitant antitumor therapy. It is worth of mention that a similar chemical strategy has been successfully adopted in the past for the standard NK₁ antagonist Aprepitant and its phosphoderivative Fosaprepitant [10,13,26]. Aprepitant and Fosaprepitant are routinely used for the treatment of CINV [16].

In the present study the pharmacological profile of Pronetupitant has been investigated in vitro and in vivo. In receptor binding experiments Pronetupitant displayed subnanomolar affinity for the NK₁ receptor, with pK_i value of 9.5. Of note in receptor binding studies the affinity of Pronetupitant for the NK₁ receptor was 3-fold higher than those reported for Netupitant (9.0 [19]), suggesting that the phosphorylation of Netupitant does not compromise the ability of the molecule to bind the NK₁ receptor. Surprisingly, this behavior is opposite to those described for Aprepitant and its pro-drug Fosaprepitant. In fact, Fosaprepitant has been reported to show ~ 30 -fold reduced binding affinity for the human NK₁ compared to Aprepitant [10,26]. As far as the selectivity of action is concerned, when screened at over 50 other G-protein coupled receptors (including NK₂ and NK₃), monoamine transporters and ion channels, Pronetupitant displayed high (~ 1000 -fold) NK₁ selectivity. This is similar to that previously reported for Netupitant [19].

In CHO_{NK1} cells SP increased intracellular calcium levels in a concentration dependent manner and with high potency. In line with previously reported data [19] this effect was counteracted by the selective and potent NK₁ antagonists Aprepitant and Netupitant, injected 24 min before SP. In this assay both Aprepitant and Netupitant behaved as insurmountable antagonists, causing a depression of the agonist maximal effect, with Aprepitant being more potent than Netupitant. Of note, the phosphoderivative pro-drug of Aprepitant, Fosaprepitant, produced effects comparable to those of Aprepitant. Considering the lower NK₁ affinity reported for Fosaprepitant compared to Aprepitant [10,26], the pharmacological effect of Fosaprepitant in this assay are likely due to its complete conversion to Aprepitant. This conversion can be ascribed to phosphatases that have been demonstrated to be expressed by CHO cells in culture [3]. Under these experimental conditions, Pronetupitant mimicked the effects of Netupitant, shifting to right the concentration response curve to SP and reducing SP maximal effect, but being ~ 10 -fold more potent than the parent compound. This gain of potency, in line with the data obtained in receptor binding experiments, suggests that Pronetupitant activity in the calcium mobilization assay cannot be simply due to its conversion to Netupitant and that Pronetupitant acts per se as an NK₁ antagonist. This hypothesis was confirmed in a subsequent series of experiments, when the time course of the NK₁ antagonist effects was investigated. Interestingly, Pronetupitant was the only NK₁ antagonist that shows similar potency values when incubated for

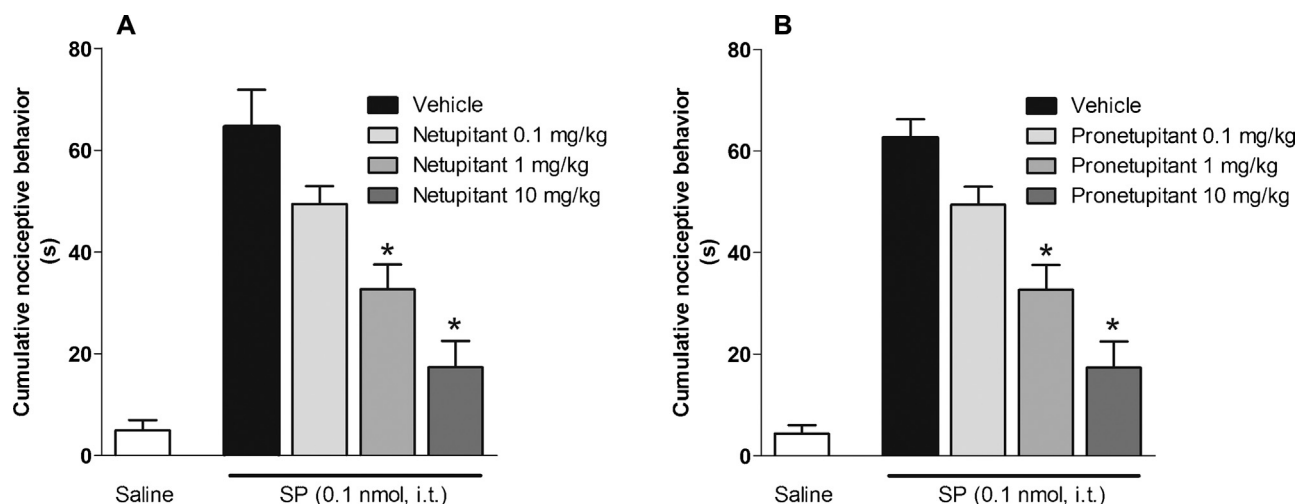


Fig. 6. SBL test in CD1 mice. Cumulative nociceptive behavior induced by i.t. administration of SP (0.1 nmol, i.t.) in mice injected i.v. (2 h pretreatment) with vehicle (saline solution with 0.05% DMSO and 0.05% Tween 80), Netupitant (0.1–10 mg/kg, panel A) or Pronetupitant (0.1–10 mg/kg, panel B). Data are mean $\pm$ SEM of 8–10 mice in each group. One-way ANOVA followed by the Dunnett's post hoc test revealed an effect of Netupitant ($F_{(3,31)} = 14.49$, panel A) and Pronetupitant ($F_{(3,33)} = 20.65$, panel B), * $p < 0.05$ vs vehicle.

Table 1

Pharmacokinetic parameter values for Pronetupitant and Netupitant in the plasma and brain of rats treated with Pronetupitant (13.16 mg/kg, i.v.).

	C_{max} (ng/ml or ng/g)	t_{max} (h)	AUC (ng/ml $\cdot$ h)	$t_{1/2}$ (h)	Cl (l/h)	AUC ratio pronetu/netu %
Plasma						
Pronetupitant	9,662	0.0166	418	0.10	31.4	1.46
Netupitant	3,077	0.083	28,700	26	0.33	
Brain						
Pronetupitant	4.33	0.25	2.11	n.c.	n.c.	0.01
Netupitant	1,335	2	19,073	11.5	0.53	

C_{max} data are expressed as mean of 3 rats.

2, 5 or 24 min before the injection of SP. On the contrary, Netupitant, Aprepitant and Fosaprepitant were almost inactive 2 min after their incubation and displayed reduced potency when incubated for 5 min compared to the 24 min incubation protocol (~3-fold for Netupitant, ~100-fold for Aprepitant and Fosaprepitant). Thus, Pronetupitant, not only behaves per se as an NK₁ antagonist more potent than Netupitant, but it is also characterized by a kinetic of interaction with the human NK₁ faster than those of Netupitant and Aprepitant. It is possible that the rapid binding and slow dissociation of Pronetupitant to NK₁ receptors prevents its rapid conversion to Netupitant by phosphatases. At the opposite, Fosaprepitant is rapidly converted to Aprepitant by phosphatases or other hydrolytic enzymes.

Different results were obtained when Pronetupitant was tested at the native NK₁ receptor expressed on the gpl. In fact, when given 15 min before SP, the behavior of Pronetupitant was comparable to those of Netupitant. Both compounds shift to right the concentration response curve to SP and evoked a significant reduction of SP maximal effects. In this assay Netupitant and Pronetupitant displayed exactly the same values of potency, suggesting that, under these experimental conditions Pronetupitant is completely converted in Netupitant. The differences between the data obtained in the calcium mobilization assay and the results from the gpl bioassay can likely be explained considering the highly exposure to the phosphatase enzymes in the native tissue than in cells culture. Of note, the presence of phosphatases has been demonstrated in the guinea pig ileum [18,25]. In line with calcium mobilization results and confirming previously reported data [19], Netupitant was less potent than Aprepitant in the gpl bioassay. When the effects of Netupitant and Pronetupitant (100 nM) and of Aprepitant and Fosaprepitant (10 nM) were tested at different times (0, 2 and 5 min)

against a fixed concentration of SP of 10 nM, Pronetupitant was the unique compound active at 2 min of incubation. This result confirmed, at the native NK₁, that Pronetupitant can behave per se as an NK₁ antagonist and that the kinetic of the interaction with the NK₁ receptor is faster for Pronetupitant compared to Netupitant and Aprepitant.

As far as the type of antagonism exerted is concerned, Pronetupitant clearly behaved as an insurmountable NK₁ antagonist. A similar behavior has already been described for other NK₁ antagonists, such as Aprepitant [17,19], Netupitant [19], Vestipitant [5] and Casopitant [4]. As discussed in details by Rizzi and colleagues [19], the insurmountable action of these molecules likely derives from a slow dissociation of the ligand receptor complex rather than from a real non competitive action. Thus, considering the chemical similarity between Pronetupitant and Netupitant, this behavior of Pronetupitant is not unexpected and can probably be ascribed to a pseudo irreversible binding to the NK₁ receptor.

In vivo, in mice subjected to the SBL test, Netupitant, given i.v. 2 h before SP, antagonized, in a dose dependent manner, the characteristic nociceptive behavior elicited by the i.t. administration of SP. This result is in line with previously reported data [19]. Under these experimental conditions, the pharmacological activity of Pronetupitant in the mouse was perfectly superimposable to those of Netupitant. Pharmacokinetic studies performed in rats demonstrated that Pronetupitant is rapidly ($t_{1/2}$ 0.10 h) and fully (AUC ratio Pronetupitant/Netupitant < 2%) converted to Netupitant and that Pronetupitant is characterized by a very low blood brain barrier permeability (C_{max} ratio brain/plasma 0.045; brain AUC ratio Pronetupitant/Netupitant 0.01%). Considering the short half-life of Pronetupitant and its low brain permeability, the in vivo activity of Pronetupitant in mice in the SBL test can completely

be ascribed to its conversion to Netupitant. Of note similar results were obtained for Aprepitant and Fosaprepitant. In fact, in rats, Fosaprepitant was rapidly converted in Aprepitant ($t_{1/2}$ 0.50 h) [13] and produced in ferrets antiemetic effects comparable to those elicited by Aprepitant [10,26].

Collectively the present study demonstrated that in vitro, in cells expressing the recombinant receptor and in tissues expressing the native receptor, Pronetupitant behaves per se as an NK₁ antagonist more potent than Netupitant and with a faster kinetic of interaction with the receptor. However, pharmacokinetics studies in rats demonstrated that in vivo Pronetupitant is rapidly and completely converted to Netupitant. In line with these findings, behavioral results obtained in mice suggest that pharmacological effects elicited by Pronetupitant and Netupitant are superimposable. Considering its high water solubility and its rapid conversion in vivo to the parental drug, Pronetupitant can represent a useful alternative to Netupitant convenient for intravenous delivery in the clinical practice.

Conflict of interest

Claudio Pietra, Emanuela Lovati and Claudio Giuliano are Helsinn employees. All the other authors declare that there is no actual or potential conflict of interest related to this study.

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