

## RESEARCH PAPER

# Pharmacological characterization of tachykinin tetrabrached derivatives

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### Keywords

tachykinins; substance P; PWT2-SP; NK<sub>1</sub> receptor; calcium mobilization; bioluminescence resonance energy transfer; guinea pig ileum; rat urinary bladder; nociceptive behaviour induced by spinal substance P; mice

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## BACKGROUND AND PURPOSE

Peptide welding technology (PWT) is a novel chemical strategy that allows the synthesis of multibrached peptides with high yield, purity and reproducibility. Using this technique, we have synthesized and pharmacologically characterized the tetrabrached derivatives of the tachykinins, substance P (SP), neurokinin A (NKA) and B (NKB).

## EXPERIMENTAL APPROACH

The following *in vitro* assays were used: calcium mobilization in cells expressing human recombinant NK receptors, BRET studies of G-protein – NK<sub>1</sub> receptor interaction, guinea pig ileum and rat urinary bladder bioassays. Nociceptive behavioural response experiments were performed in mice following intrathecal injection of PWT2-SP.

## KEY RESULTS

In calcium mobilization studies, PWT tachykinin derivatives behaved as full agonists at NK receptors with a selectivity profile similar to that of the natural peptides. NK receptor antagonists display similar potency values when tested against PWT2 derivatives and natural peptides. In BRET and bioassay experiments PWT2-SP mimicked the effects of SP with similar potency, maximal effects and sensitivity to aprepitant. After intrathecal administration in mice, PWT2-SP mimicked the nociceptive effects of SP, but with higher potency and a longer-lasting action. Aprepitant counteracted the effects of PWT2-SP *in vivo*.

## CONCLUSIONS AND IMPLICATIONS

The present study has shown that the PWT technology can be successfully applied to the peptide sequence of tachykinins to generate tetrabrached derivatives characterized with a pharmacological profile similar to the native peptides. *In vivo*, PWT2-SP displayed higher potency and a marked prolongation of action, compared with SP.

## Abbreviations

DMF, dimethylformamide; HATU, [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NKA, neurokinin A; NKB, neurokinin B; NMM, 4-methylmorpholine; PWT, peptide welding technology; SBL, scratching, biting and licking; SP, substance P

## Introduction

Most currently available pharmaceutical agents are orally available, low MW molecules. This formulation has undoubtedly pharmacokinetic advantages, but quite often suffer from limited target selectivity and consequent side effects. In contrast, peptides display extraordinarily high selectivity of action, but at the expense of a poor pharmacokinetic profile; including low bioavailability, poor barrier penetrance and high susceptibility to enzymatic degradation. One strategy to improve the pharmacokinetic properties of peptides, particularly their poor metabolic stability, is the use of multibranched peptides. However, the synthesis of these molecules is rather difficult, and in general, characterized by low yield and purity of the final product.

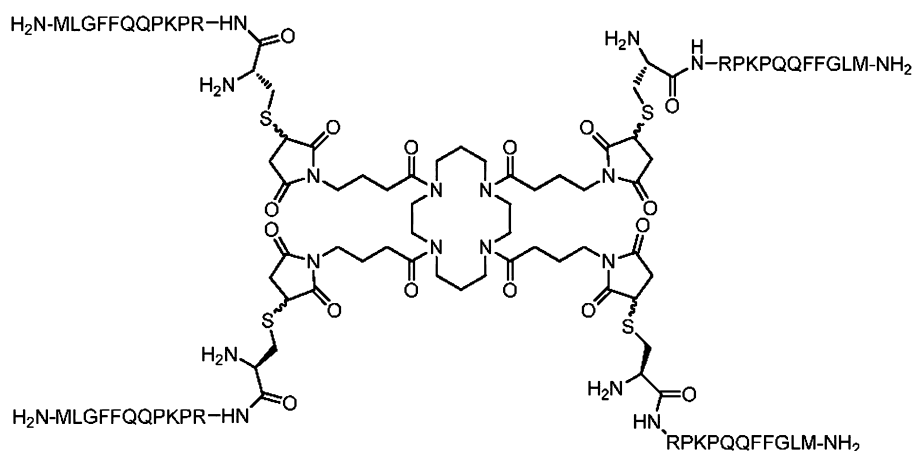
We have recently developed an innovative chemical approach, referred to as peptide welding technology (PWT), which allows the synthesis of multibranching peptides with excellent high yield, purity and reproducibility. With this approach, three different tetrabranching derivatives of the neuropeptide, nociceptin/orphanin FQ, have been synthesized and pharmacologically characterized *in vitro* and *in vivo*. *In vitro*, these compounds retained the biological activity of the natural peptide both at human recombinant and native receptors. In mice, these peptides showed higher potency and a marked prolongation of action (Rizzi *et al.*, 2014). Based on these results, in the present study, we sought to investigate the pharmacological profile of PWT derivatives of tachykinins. Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) share a common C-terminal sequence Phe-X- Gly-Leu-Met that accounts for their biological activity. Tachykinins bind to and activate three different GPCRs: NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> receptors (Regoli *et al.*, 1994; receptor nomenclature follows Alexander *et al.*, 2013, see also Neubig *et al.*, 2003). Tachykinins are neurotransmitters widely distributed both in the central and in the peripheral nervous systems where they control several biological functions, such as pain and migraine, nausea and vomiting, mood and anxiety, drug abuse and inflammatory conditions of the gastrointestinal tract (Douglas *et al.*, 2014).

In the present study, PWT has been used to generate tetrabranching derivatives of the tachykinins: PWT2-SP, PWT2-NKA and PWT2-NKB. The chemical structure of PWT2-SP is shown in Figure 1. These compounds were evaluated pharmacologically for their ability to stimulate calcium mobilization in CHO cells stably expressing the human recombinant NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> receptors. Moreover PWT2-SP has been further characterized *in vitro* for its ability to promote the association of the NK<sub>1</sub> receptor with G-protein in BRET experiments and to evoke contractions of the guinea pig ileum and rat urinary bladder in bioassay experiments. Finally, PWT2-SP has been assayed *in vivo* after intrathecal (i.t.) administration in the mouse for its ability to evoke a set of nociceptive behaviours such as scratching, biting and licking (SBL) responses.

## Methods

### Synthesis of PWT tachykinin derivatives

These were prepared using a convergent synthetic approach. As an example, the synthesis of PWT2-SP is described. [Cys<sup>0</sup>]SP was synthesized using a solid phase methodology with an automatic solid phase peptide synthesizer Syro II (Biotage, Uppsala, Sweden) and Fmoc/tBu chemistry (Benoiton, 2005). The resin 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA (Rink amide MBHA resin) was used as a solid support. The resin was treated with 40% piperidine/dimethylformamide (DMF) and linked with Fmoc-Met-OH by using [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) as the coupling reagent. The following Fmoc amino acids were sequentially coupled to the growing peptide chain: Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Phe-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Cys(Trt)-OH. All the Fmoc amino acids (4 equiv) were coupled to the growing peptide chain using HATU (4 equiv) in DMF in the presence of an equimolar concentration of



**Figure 1**

Chemical formula of PWT2-SP.

4-methylmorpholine (NMM). The coupling reaction time was 1 h. To improve the analytical profile of the crude peptide, capping with acetic anhydride (0.5 M in DMF) in the presence of NMM (0.25 M in DMF) (3:1 v/v; 2 mL per 0.2 g of resin) was performed at any step. 40% piperidine in DMF was used to remove the Fmoc. The protected peptide-resin was treated with reagent B (Solé and Barany, 1992) [trifluoroacetic acid (TFA)/H<sub>2</sub>O/phenol/triisopropylsilane 88: 5:5:2; v/v; 10 mL 0.2·g<sup>-1</sup> of resin] for 1.5 h at room temperature. After filtration of the resin, the solvent was concentrated *in vacuo* and the residue triturated with ether. Crude [Cys<sup>0</sup>]SP was purified by preparative reversed-phase HPLC using a Waters Delta Prep 3000 system (Milford, MA, USA) with a Jupiter C<sub>18</sub> column (250 × 30 mm, 300 Å, 15 µm spherical particle size). The column was perfused at a flow rate of 20 mL·min<sup>-1</sup> with a mobile phase containing solvent A (5%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 70% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min for the elution of peptides. Purified [Cys<sup>0</sup>]SP was reacted in solution with PWT2 core in a classical thio-Michael reaction using experimental conditions previously optimized for the synthesis of nociceptin/orphanin FQ tetrabrached derivatives. The reaction proceeded rapidly at room temperature in acetonitrile/water and with the presence of a weak base (NaHCO<sub>3</sub>), it was complete in approximately 4 min, and displayed an impressive yield (virtually 100%). Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Analytical purity of [Cys<sup>0</sup>]SP and PWT2-SP were determined using a Luna C<sub>18</sub> column (4.6 × 100 mm, 3 µm particle size) with the solvent system mentioned earlier (solvents A and B) programmed at a flow rate of 0.5 mL·min<sup>-1</sup> using a linear gradient from 0 to 80% B over 25 min. Final product showed ≥95% purity when monitored at 220 nm. Similar procedures were applied for the synthesis of PWT2-NKA and PWT2-NKB.

### Calcium mobilization assay

Cells stably expressing the human NK<sub>1</sub> receptor were a generous gift from the laboratories of Prof. T. Costa (ISS, Rome, Italy), while cells stably expressing the human NK<sub>2</sub> and NK<sub>3</sub> receptors were a gift from Prof. T.W. Schwartz (University of Copenhagen, Denmark). CHO<sub>NK1</sub>, CHO<sub>NK2</sub> and CHO<sub>NK3</sub> cells were maintained in DEMEM/F-12 medium supplemented with 10% FBS, 100 U·mL<sup>-1</sup> penicillin and 100 µg·mL<sup>-1</sup> streptomycin and 200 µg·mL<sup>-1</sup> G418, and cultured at 37°C in 5% CO<sub>2</sub> humidified air. Cells were seeded at a density of 50 000 cells per well into 96-well black, clear-bottom plates. The following day, the cells were incubated with medium supplemented with 2.5 mM probenecid, 3 µM 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 µL·well<sup>-1</sup> of assay buffer (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 µM Brilliant Black (Sigma Aldrich, St. Louis, MO, USA) was added. Concentrated solutions (1 mM) of SP and PWT2-SP were made in bi-distilled water and stored at -20°C. NKA, PWT2-NKA, NKB and PWT2-NKB were solubilized in 0.1 M Na<sub>2</sub>CO<sub>3</sub> at a final concentration of 1 mM. Aprepitant, GR159897 and SB222200 (10 mM) were dissolved in DMSO. Stock solutions were kept at -20°C until use. Serial dilutions were made in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction

V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA, USA), fluorescence changes were measured. Online additions were carried out in a volume of 50 µL per well. To facilitate drug diffusion into the wells, the present studies were performed at 37°C, and in antagonism experiments, three cycles of mixing (25 µL from each well moved up and down three times) were performed immediately after antagonist injection to the wells. Antagonists were injected into the wells 24 min before adding agonists. Maximum change in fluorescence, expressed as percentage over the baseline fluorescence, was used to determine agonist response.

### BRET assay

Mouse fibroblasts MB19tsA cells, 2B2 clone from the laboratory of Prof. O.H. Onaran (Ankara University, Ankara, Turkey), were cultured in a 50% mixture of DMEM and F12, containing 10% FBS in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cell lines with stable co-expression of *Renilla* luciferase tagged human NK<sub>1</sub> receptor and the *Renilla* green fluorescent protein fused Gβ<sub>1</sub> were obtained by infecting cells sequentially with retroviruses encoding each single fusion protein followed by selection with G418 (500 µg·mL<sup>-1</sup>) in combination with hygromycin B (100 µg·mL<sup>-1</sup>). The BRET assay of G-protein coupling was performed using enriched membranes from transfected cells, prepared by differential centrifugation (Vachon *et al.*, 1987) and stored in aliquots at -80°C before use. Membranes (5 µg·100 µL<sup>-1</sup>) in PBS, MgCl<sub>2</sub> 5 mM, were first preincubated with 500 nM coelenterazine for 10 min in white 96-well Optiplates (Perkin-Elmer, Waltham, MA, USA). Next, increasing concentrations of agonists were added, and the incubation proceeded for an additional 5 min. The plate was loaded into a luminometer (VICTOR light, Perkin-Elmer) and the BRET signals were measured and analysed as described in Molinari *et al.* (2010). Results are expressed as BRET ratio (cps at 510 nm·cps<sup>-1</sup> at 475 nm).

### Isolated tissue bioassays

All animal care and experimental procedures complied with the European Communities Council directives (86/609/EEC) and national regulations (D.L. 116/92) and were approved by the Ethical Committee of the University of Ferrara. All experimental procedures adopted for *in vivo* and *ex vivo* studies were as humane as possible, complied with the, and have been reported according to ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The total number of animals used in the experiments described in this study was 8 guinea pigs, 8 rats and 60 mice.

Male albino guinea pigs (300–350 g, Pamapaloni, Italy) were housed in 300 × 570 × 155 mm cages (Tecniplast, MN, Italy), under standard conditions (22°C, 55% humidity, 12 h light–dark cycle, lights on 07:00 h) with food (4RE, Mucedola, Italy) and water *ad libitum* for at least 7 days before the experiment. 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, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, CaCl<sub>2</sub> 2.5, glucose 10, hexamethonium bromide 2.2 µM and benadril 0.3 µM) oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The

temperature was set at 37°C. A resting tension of 1 g was applied to the tissues. Tissue contractions were measured isometrically with a force transducer (FT03, Grass Instruments, Warwick, RI, USA) and recorded on a multichannel chart recorder (Linseis Model L2005, Linseis Messgeraete GmbH, Selb, Germany). After an equilibration period of about 60 min, to check the responsiveness of the preparation, the contractile effect of carbachol 100 µM was recorded. After approximately 20 min, atropine 1 µM was added to the medium to prevent the effects of ACh release induced by activating presynaptic NK<sub>3</sub> receptors (Laufer *et al.*, 1985) and 20 min later, a cumulative concentration–response curve to SP or PWT2-SP were constructed. In antagonism experiments the concentration–response curve to SP or PWT2-SP were tested in the absence or presence of aprepitant (1 nM) with 15 min pre-incubation time.

Male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 280–330 g were housed in 425 × 266 × 155 mm cages (Tecniplast, MN, Italy), under standard conditions (22°C, 55% humidity, 12 h light–dark cycle, lights on 07:00 h) with food (4RF) and water *ad libitum* for at least 7 days before the experiment. These experiments were performed following the protocol described by Meini and Maggi (2010). Briefly, after killing the animal via isoflurane overdose, the bladder was taken and dissected longitudinally in two parts, and then suspended in 5 mL organ baths containing Krebs solution oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. A resting tension of 0.5 g was applied to the tissues and bath solution was replaced every 20 min. Tissue contractions were measured isotonicly by means of Basile strain gauge transducers and recorded with a PC-based acquisition system (Power Lab, 4/25, ADInstruments, NSW, Australia). After an equilibration period of about 60 min, 100 µM carbachol was added to the bath solution to induce the maximal contractile response for use as a reference value. Subsequently, cumulative concentration–response curves (0.5 log unit steps) to SP and PWT2-SP were constructed. All experiments were performed in the presence of the NK<sub>2</sub> receptor antagonist SR 48968 (1 µM). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

### Nociceptive behaviours induced by i.t. PWT2-SP in mice

Male CD-1 mice (weight 20–25 g, Harlan, Udine, Italy) were housed in 425 × 266 × 155 mm cages (Tecniplast), under standard conditions (22°C, 55% humidity, 12 h light–dark cycle, lights on 07:00 h) with food (4RF) and water *ad libitum* for at least 3 days before experiments began. Each animal was used only once. SP (100 pmol) and PWT2-SP (2.5–250 pmol) were given spinally. I.t. injections (5 µL per mouse) were performed according to the procedure described by Hylden and Wilcox (1980) and routinely used in our laboratory (Rizzi *et al.*, 2006). Approximately, 45 min before i.t. injection, mice were adapted to an individual plastic cage, which served as the observation chamber. The animals were randomly assigned to treatments (saline, SP and PWT2-SP) and individually observed for 30 min. The total time (s) spent displaying the following behaviours was measured: hind limb scratching directed towards the flank; biting or licking of the

fore and hind paws; biting or licking of the tail (Takahasi *et al.*, 1987). Aprepitant (1 mg·kg<sup>-1</sup>, i.p.) was solubilized in 5% DMSO and administered 2 h before PWT2-SP (25 pmol, i.t.). All experiments started at 09:00 h.

### Data analysis

Data from *in vitro* experiments are expressed as means ± SEM of *n* experiments. Non-linear regression analysis using GraphPad Prism software (v.4.0; GraphPad Software, Inc, La Jolla, CA, USA) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Agonist potencies were expressed as pEC<sub>50</sub>. In inhibition response experiments (i.e. increasing concentrations of antagonist vs. a fixed concentration of agonist) the antagonist potency was expressed as pIC<sub>50</sub>. A fixed concentration of aprepitant was also challenged against the agonist concentration–response curve. In calcium mobilization and guinea pig ileum experiments the antagonist displayed insurmountable behaviour, thus according to Gaddum *et al.* (1955), the following equation was used to derive antagonist potency:

$$pK_B = \log_{10}[(\text{slope} - 1)/(B)]$$

In practice, equiactive concentrations of the agonist in the absence and presence of an insurmountable antagonist [ (B) ] are compared in a double reciprocal plot describing a straight line. In contrast, in rat urinary bladder experiments aprepitant displayed a competitive behaviour and its antagonist potency was derived from following equation:

$$pK_B = \log_{10}[(CR - 1)/(B)]$$

where *CR* is the ratio of potency between the agonist EC<sub>50</sub> in the presence and absence of antagonist and (*B*) is the antagonist molar concentration.

*In vivo* data are expressed as mean ± SEM of *n* animals. Data were analysed using Student's *t*-test for unpaired data or one-way ANOVA followed by Dunnett's *post hoc* test. Differences were considered statistically significant when *P* < 0.05.

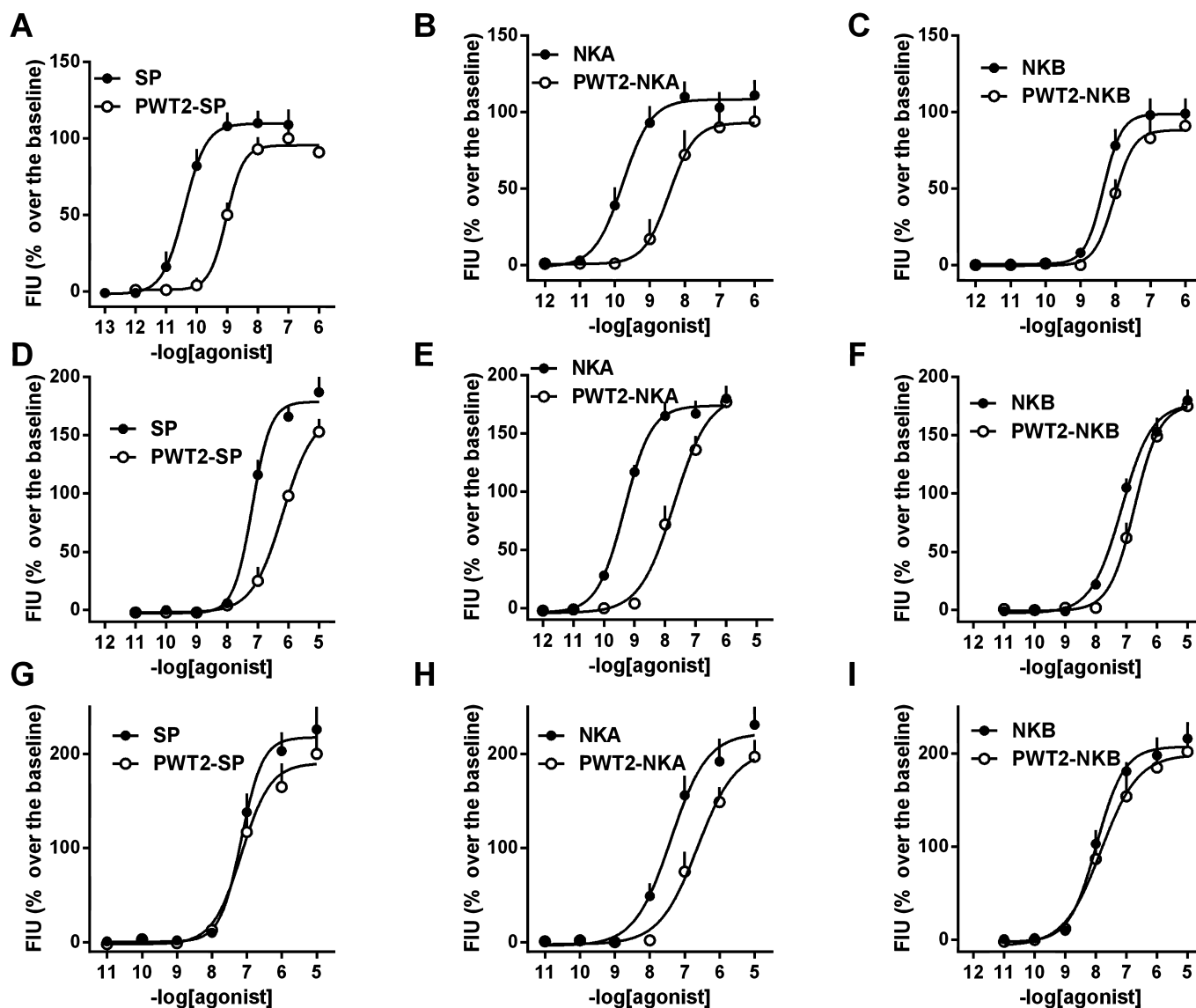
### Materials

GR159897, SB222200 atropine and carbachol were purchased from Sigma Chemical Co. (St Louis, MO, USA). NKA and NKB were purchased from Tocris Biosciences (Bristol, UK). SP was synthesized and purified in our laboratories. Aprepitant was a generous gift from Dr C Pietra (Helsinn, Lugano, CH).

## Results

### Calcium mobilization studies

In a first series of experiments the effects of PWT2-SP, PWT2-NKA and PWT2-NKB were assessed in CHO cells expressing the human NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> receptors and compared with those elicited by the natural tachykinins SP, NKA and NKB. In addition the actions of the peptides used in the synthesis of PWT molecules, that is [Cys<sup>0</sup>]SP, [Cys<sup>0</sup>]NKA and [Cys<sup>0</sup>]NKB, were also evaluated in parallel experiments. The effects of [Cys<sup>0</sup>] derivatives of tachykinins were always identical to those of the natural peptides and, for the sake of clarity, these results have not been displayed in the Figures. As shown in Figure 2, in CHO<sub>NK1</sub> cells, SP stimulated calcium mobiliza-



**Figure 2**

Calcium mobilization assay performed in CHO cells expressing NK<sub>1</sub> (panels A, B and C), NK<sub>2</sub> (panels D, E and F) and NK<sub>3</sub> (panels G, H and I) receptors. Concentration–response curve to SP and PWT2-SP (panels A, D and G), NKA and PWT2-NKA (panels B, E and H), and NKB and PWT2-NKB (panels C, F and I). Data are mean ± SEM of four experiments performed in duplicate.

tion in a concentration-dependent manner with high potency ( $pEC_{50} = 10.40$ ) and an  $E_{max}$  value of  $109 \pm 8\%$  over the basal levels. NKA and NKB mimicked SP action with similar maximal effects, but lower potency (by approximately 10- and 100-fold respectively). PWT2-SP and PWT2-NKA stimulated intracellular calcium mobilization producing similar maximal effects as the natural peptides, but were approximately 30-fold less potent. In contrast, the concentration–response curves to NKB and PWT2-NKB were virtually superimposable (Figure 2, panels A, B and C). In CHO<sub>NK2</sub> cells NKA increased intracellular calcium levels in a concentration-dependent manner, with a  $pEC_{50}$  value of 9.32 and an  $E_{max}$  value of  $174 \pm 11\%$  over the basal. SP and NKB produced maximal effects similar to those of NKA, but were approximately 100-fold less potent. PWT2-NKA elicited

similar maximal effects as the natural peptide, but was 30-fold less potent. Similar results were obtained with PWT2-SP and PWT2-NKB, but the reduction in potency for these compounds in comparison with the natural peptides was limited to 10- and threefold respectively (Figure 2, panels D, E and F). In CHO<sub>NK3</sub> cells, NKB increased intracellular calcium levels in a concentration-dependent manner with a  $pEC_{50}$  value of 7.96 and an  $E_{max}$  value of  $207 \pm 18\%$ . SP and NKA evoked similar maximal effects as NKB, but were significantly less potent. In this cell line, PWT tachykinin derivatives elicited concentration–response curves superimposable to those of the natural peptides with the exception of PWT2-NKA that was sixfold less potent than NKA (Figure 2, panels G, H and I). Results obtained in this series of experiments have been summarized in Table 1.

**Table 1**

Potencies and maximal effects of natural tachykinins and their PWT derivatives in calcium mobilization experiments performed in CHO cells expressing human NK receptors

	NK <sub>1</sub>		NK <sub>2</sub>		NK <sub>3</sub>	
	pEC <sub>50</sub> (CL <sub>95%</sub> )	E <sub>max</sub>	pEC <sub>50</sub> (CL <sub>95%</sub> )	E <sub>max</sub>	pEC <sub>50</sub> (CL <sub>95%</sub> )	E <sub>max</sub>
SP	10.40 (9.93–10.87)	109 ± 8%	7.18 (6.99–7.37)	178 ± 19%	7.15 (7.01–7.29)	217 ± 30%
PWT2-SP	9.02 (8.85–9.19)	95 ± 2%	6.17 (5.62–6.72)	168 ± 11%	7.15 (6.90–7.45)	190 ± 21%
NKA	9.70 (9.32–10.08)	108 ± 10%	9.32 (9.05–9.58)	174 ± 11%	7.39 (6.81–7.97)	222 ± 21%
PWT2-NKA	8.44 (7.79–9.09)	93 ± 10%	7.70 (7.06–8.36)	183 ± 10%	6.63 (6.03–7.23)	204 ± 18%
NKB	8.34 (8.13–8.55)	99 ± 10%	7.15 (6.81–7.49)	177 ± 9%	7.96 (7.62–8.30)	207 ± 18%
PWT2-NKB	8.03 (7.82–8.24)	88 ± 11%	6.73 (6.22–7.24)	176 ± 13%	7.82 (7.25–8.39)	199 ± 17%

Data are mean ± SEM of at least four experiments performed in duplicate. CL<sub>95%</sub>, 95% confidence limits.

The stimulatory effects elicited by PWT tachykinin derivatives were challenged with selective NK receptor antagonists; aprepitant (Kramer *et al.*, 1998) for NK<sub>1</sub>, GR159897 (Beresford *et al.*, 1995) for NK<sub>2</sub>, and SB222200 (Sarau *et al.*, 2000) for the NK<sub>3</sub> receptors. Antagonists were evaluated in inhibition response experiments, where a fixed concentration of agonist, approximately corresponding to its EC<sub>80</sub>, was challenged against increasing concentrations of antagonist. In CHO<sub>NK1</sub> cells, aprepitant produced a concentration-dependent inhibition of the stimulatory actions of both SP (1 nM) and PWT2-SP (10 nM) with similar potency values (pIC<sub>50</sub> = 8.57 and 8.53, respectively, Figure 3, panels A and B). GR159897 and SB222200 up to 1 μM did not affect PWT2-SP responses. In CHO<sub>NK2</sub> cells, GR159897 blocked the stimulatory effects of NKA (1 nM) and PWT2-NKA (100 nM) with pIC<sub>50</sub> values of 8.15 and 8.11 respectively (Figure 3, panels C and D). Aprepitant and SB222200 up to 1 μM did not affect PWT2-NKA responses. Finally, in CHO<sub>NK3</sub> cells SB222200 blocked the stimulatory effects of NKB and PWT2-NKB (both at 100 nM) with pIC<sub>50</sub> values of 7.01 and 7.00, respectively (Figure 3, panels E and F), while aprepitant and GR159897 were inactive. The results of this series of antagonist experiments have been summarized in Table 2.

When the concentration–response curve to SP was carried out in the presence of a fixed concentration of aprepitant (1 nM), this antagonist produced a slight rightward shift of the curve associated with a large reduction in agonist maximal effects (Figure 4, panel A). A pK<sub>B</sub> value of 9.66 was derived from these experiments. Similarly, aprepitant slightly shifted to right the concentration–response curve to PWT2-SP, reducing its maximal effects and with a pK<sub>B</sub> value of 9.46 (Figure 4, panel B).

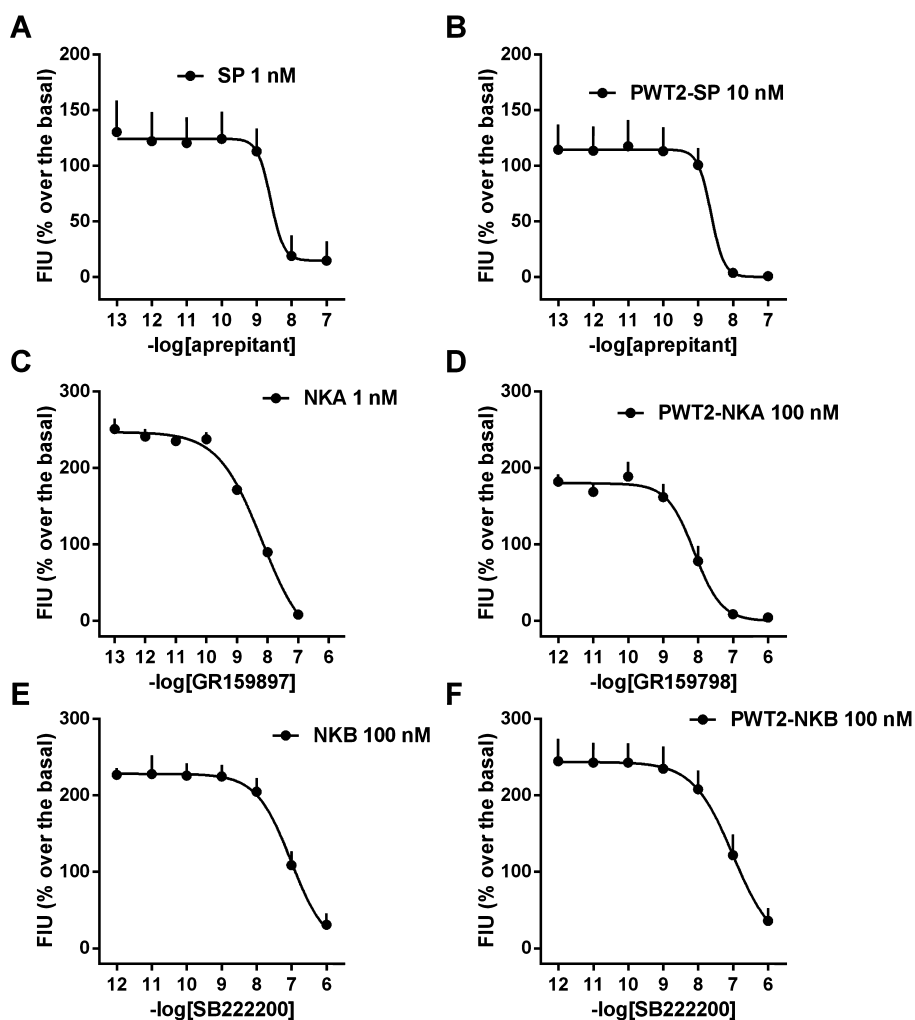
### BRET studies

In the BRET assay, SP produced a concentration-dependent stimulation of NK<sub>1</sub> receptor–G-protein association with a maximal effect of 0.53 ± 0.01 and a pEC<sub>50</sub> of 9.07 (CL<sub>95%</sub> 8.73–9.42). PWT2-SP mimicked the action of the natural peptide with a concentration–response curve not different from that of SP (Figure 5).

### Isolated tissue bioassays

In the guinea pig ileum, SP produced a concentration-dependent contraction with a pEC<sub>50</sub> and E<sub>max</sub> value of 8.50 (CL<sub>95%</sub> 8.19–8.81) and 3.25 ± 0.37 g respectively. PWT2-SP mimicked the contractile effects of SP with similar potency [8.64 (CL<sub>95%</sub> 8.48–8.78)] and maximal effects (3.11 ± 0.16 g, Figure 6, panel A). Panel B of Figure 6 displays typical tracings of the contractile effects of SP and PWT2-SP in the guinea pig ileum. Immediately after agonist injection the tissue rapidly responded. The contractile responses induced by SP and PWT2-SP were very similar. In contrast, after washing, SP-induced contraction rapidly disappeared with only a few seconds needed to return to baseline, while the contractile effect evoked by PWT2-SP was less readily washed out and more than 1 min was required to return to baseline. In order to investigate the receptor involved in the contractile effects of SP and PWT2-SP in the guinea pig ileum their actions were challenged with the known NK<sub>1</sub> receptor antagonist aprepitant. As shown in Figure 6, panels C and D, aprepitant (1 nM) produced a rightward shift in the concentration–response curves to SP and PWT2-SP along with a significant reduction in their maximal effects. From these experiments pK<sub>B</sub> values of 10.41 and 10.21 were derived for aprepitant versus SP and PWT2-SP respectively.

In the rat urinary bladder, SP and PWT2-SP produced superimposable concentration–response curves with similar potencies and maximal effects (Figure 7, panel A). Panel B of Figure 7 displays typical tracings of the contractile effects of SP and PWT2-SP in the rat urinary bladder. Immediately after SP injection, the tissue rapidly contracted reaching the plateau in less than 2 min. After washing, this rapidly returned to baseline. In contrast, the contraction elicited by PWT2-SP was slower to reach a plateau, taking more than 6 min, and essentially resistant to washing; Aprepitant (1 μM) did not modify the contractile effect of SP and PWT2-SP in the rat urinary bladder (data not shown). At 10 μM, the antagonist produced a slight rightward shift in the concentration–response curves to SP and PWT2-SP without significantly modifying their maximal effects. From these experiments, pK<sub>B</sub> values of 5.42 and 5.31 were derived for aprepitant versus SP and PWT2-SP (Figure 7, panels C and D).



**Figure 3**

Calcium mobilization assay performed in CHO cells expressing NK<sub>1</sub> (panels A and B), NK<sub>2</sub> (panels C and D), and NK<sub>3</sub> (panels E and F) receptors. Inhibition–response curve to NK receptor antagonists against the stimulatory effect of natural tachykinins (panels A, C and E) and their PWT derivatives (panels B, D and F). Data are mean ± SEM of four experiments performed in duplicate.

**Table 2**

pIC<sub>50</sub> values of NK receptor antagonists obtained in inhibition response experiments against PWT2-NK derivatives in calcium mobilization experiments performed in CHO cells expressing human NK receptors

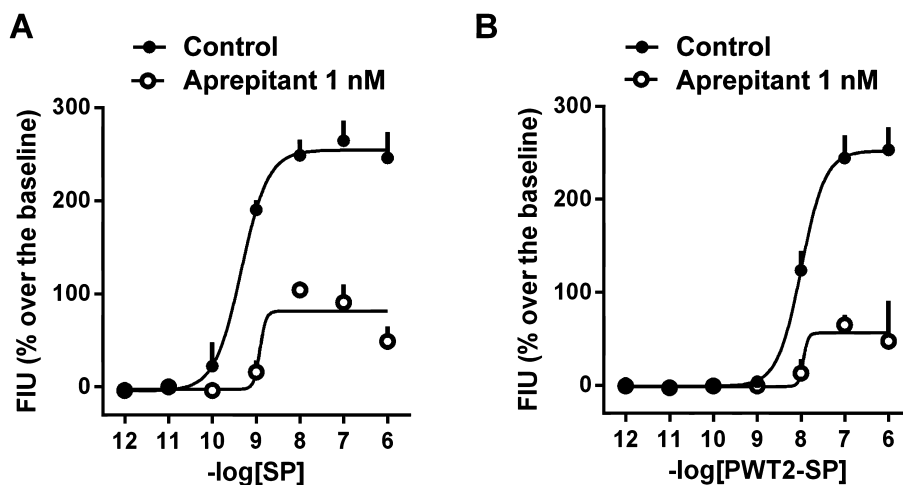
Receptor agonist	NK <sub>1</sub> PWT2-SP	NK <sub>2</sub> PWT2-NKA	NK <sub>3</sub> PWT2-NKB
Aprepitant	8.53 (8.16–8.90)	<6	<6
GR159897	<6	8.11 (7.59–8.63)	<6
SB222200	<6	<6	7.00 (6.60–7.40)

Data are mean (CL<sub>95%</sub>) of at least four experiments performed in duplicate.

### *Nociceptive behaviours induced by i.t. PWT2-SP in mice*

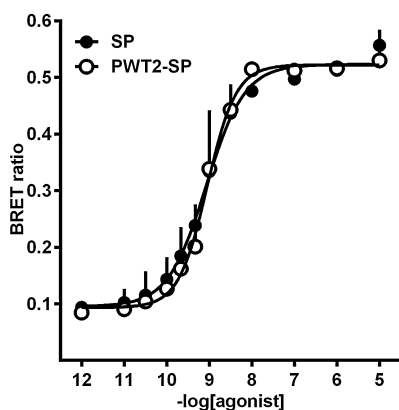
Previous studies (Hylden and Wilcox, 1981) recently replicated in our laboratories (Rizzi *et al.*, 2012) demonstrated that the i.t. injection of SP in mice elicits a typical nociceptive

behaviour consisting of scratching (S), biting (B) and licking (L). In line with these findings, in the present experiments SP (100 pmol; i.t.) elicited a typical SBL response. The effect of SP peaked immediately after injection and rapidly disappeared after 10 min (Figure 8, panel A). Under the same



**Figure 4**

Calcium mobilization assay performed in CHO cells expressing NK<sub>1</sub> receptors. Concentration–response curve to SP (panel A) and PWT2-SP (panel B) in absence and in presence of 1 nM aprepitant. Data are mean  $\pm$  SEM of three experiments performed in duplicate.



**Figure 5**

BRET assay performed in fibroblasts stably expressing *Renilla* luciferase tagged NK<sub>1</sub> receptors and *Renilla* green fluorescent protein fused G $\beta$ <sub>1</sub> protein. Concentration–response curves to SP and PWT2-SP. Data are mean  $\pm$  SEM of three experiments performed in duplicate.

experimental conditions, PWT2-SP (2.5–250 pmol) dose-dependently elicited the SBL response mimicking the effect of SP. PWT2-SP was more potent and efficacious and produced longer-lasting effects. Comparing the effects of 100 pmol SP and 25 pmol PWT2-SP (these doses contain the same number of peptide sequences) indicates the effect elicited by PWT2-SP is approximately sixfold larger than that of the natural peptide. Moreover the action of SP was no longer evident 10 min after injection while that of PWT2-SP lasted for the entire duration of the experiment, 30 min (Figure 8, panel A). In order to investigate the receptor involved in the nociceptive effect of PWT2-SP, a series of experiments were performed in mice treated with vehicle or 1 mg·kg<sup>-1</sup> aprepitant. At this dose the antagonist did not produce, *per se*, any nociceptive behaviour (data not shown). However, when aprepitant was

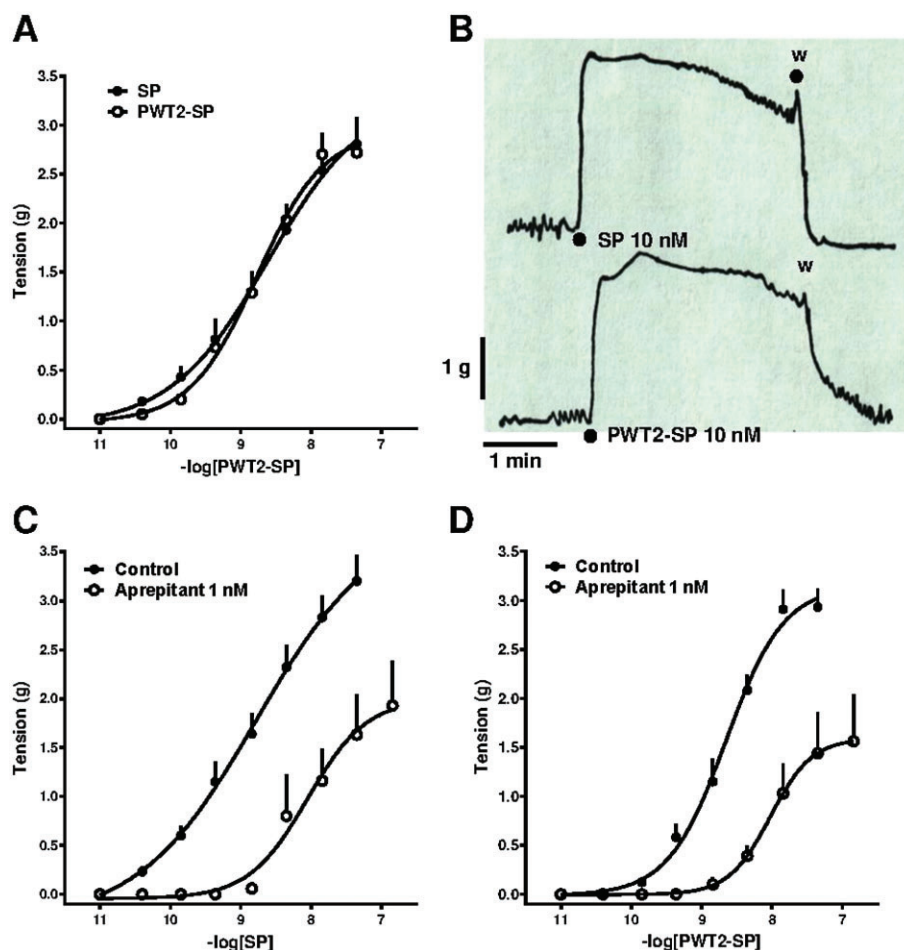
tested against 25 pmol PWT2-SP, it significantly inhibited agonist-induced nociceptive behaviour (Figure 8, panels C and D).

## Discussion and conclusions

In the present study, the pharmacological profile of tachykinin tetrabranch derivatives produced using PWT technology have been investigated. *In vitro*, in the calcium mobilization assay, PWT2-SP, PWT2-NKA and PWT2-NKB behaved as full agonists at human recombinant NK receptors maintaining a profile of selectivity in common with the natural peptides. The potency of PWT derivatives compared with natural tachykinins was similar or reduced depending on both peptide sequence and receptor. In BRET experiments investigating NK<sub>1</sub> receptor / G-protein interactions, both PWT2-SP and SP produced results that were superimposable in terms of maximal effects and potency. Moreover, similar findings were obtained in bioassay experiments at native NK<sub>1</sub> receptors expressed in the guinea pig ileum and the rat urinary bladder. Finally, *in vivo* after i.t. injection in mice, PWT2-SP mimicked the nociceptive effect of SP, but with higher potency and effectiveness along with a prolonged duration of action. The *in vitro* and *in vivo* effects of PWT2-SP were sensitive to the NK<sub>1</sub>-selective antagonist aprepitant. Thus, the present study demonstrated that the PWT technology can be successfully applied to tachykinin peptide sequences to obtain novel ligands that display an *in vitro* pharmacological profile similar to natural tachykinins, but display higher potency and longer-lasting effects *in vivo*.

PWT technology has recently been developed and validated by generating tetrabranch derivatives of the opioid-like neuropeptide N/OAQ, using three different maleimide functionalized cores: (Lys)<sub>2</sub>-Lys-NH<sub>2</sub> for PWT1-N/OAQ, Cyclam for PWT2-N/OAQ, and (Lys)<sub>2</sub>-ethylendiamine for PWT3-N/OAQ. In *in vitro* and *in vivo* pharmacological studies PWT2-N/OAQ generated the best results in terms of maintain-





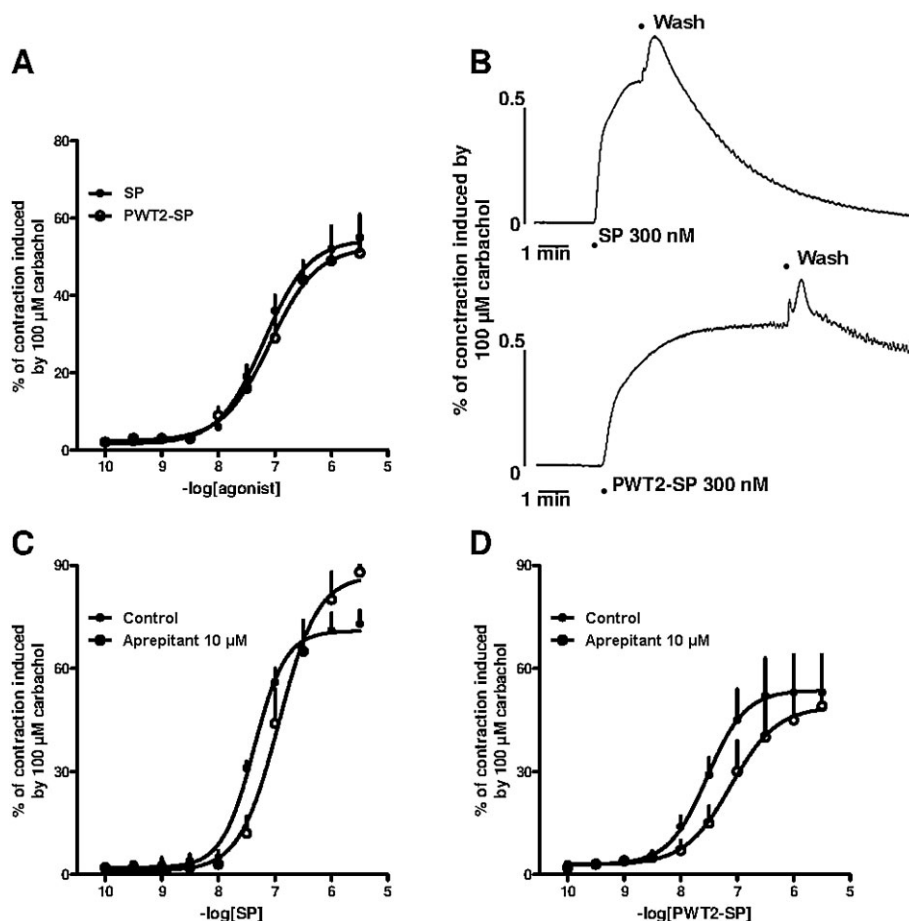
**Figure 6**

Guinea pig ileum bioassay. Concentration–response curves to SP and PWT2-SP (panel A) and representative tracings of tissue contraction in response to SP and PWT2-SP (panel B). Concentration–response curve to SP (panel C) and PWT2-SP (panel D) in absence and in presence of aprepitant 1 nM. Data are mean  $\pm$  SEM of four experiments performed in duplicate.

ing the selectivity of the natural peptide sequence along with higher *in vivo* potency and longer-lasting effects (Rizzi *et al.*, 2014). Based on these results, the PWT2 core was selected for generating PWT tachykinin derivatives. In addition to the core used for the design of multivalent ligands another crucial issue is the selection of the attachment point (Shonberg *et al.*, 2011). As noted earlier, tachykinins share a common C-terminal sequence Phe-X-Gly-Leu-Met that accounts for their biological activity (Regoli *et al.*, 1994; Douglas *et al.*, 2014). Thus, we decided to use the N terminal of the peptide sequence as attachment point for the Cys residue crucial for PWT reaction. Therefore [Cys<sup>0</sup>]SP, [Cys<sup>0</sup>]NKA, and [Cys<sup>0</sup>]NKB were synthesized. A large body of evidence suggests that the N terminal of SP can be utilized to generate conjugated molecules without losing the ability of the peptide to bind and activate the NK<sub>1</sub> receptor. This strategy has been successfully used for delivery of several different toxins including saporin (Mantyh *et al.*, 1997), diphtheria (Benoliel *et al.*, 1999), cholera (Caudle *et al.*, 2007) and, more recently, botulinum toxin (Mustafa *et al.*, 2013) into cells expressing NK<sub>1</sub> receptors. In line with these findings the present results obtained in calcium mobilization experiments

demonstrated that the pharmacological profile of [Cys<sup>0</sup>]SP, [Cys<sup>0</sup>]NKA, and [Cys<sup>0</sup>]NKB are identical to those for the natural tachykinins in terms of efficacy, potency, and selectivity of action. Thus [Cys<sup>0</sup>]tachykinins were linked together with the PWT2 core using a thio-Michael reaction. As in our results obtained using the N/OFG sequence, the reaction proceeded in a few minutes and was characterized by an extremely high yield and purity of the final products: PWT2-SP, PWT2-NKA and PWT2-NKB.

In the calcium mobilization assay performed with CHO cells stably transfected with human recombinant NK receptors, the natural tachykinins SP, NKA and NKB behaved as full agonists, with the following rank–order potency: SP > NKA > NKB, NKA > SP = NKB, NKB > NKA > SP at NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> receptors respectively. These findings are fully consistent with results obtained using bioassay techniques and animal tissues (Regoli *et al.*, 1988; 1994) and cells expressing recombinant human receptors (Rizzi *et al.*, 2012). PWT2 tachykinin derivatives mimicked the stimulatory effects of the natural peptides with similar maximal effects in the three cell lines. Thus all PWT2 tachykinins behaved as full agonists. This mirrors our previous work with N/OFG PWT derivatives (Rizzi *et al.*,



**Figure 7**

Rat urinary bladder bioassay. Concentration–response curves to SP and PWT2-SP (panel A) and representative tracings of tissue contraction in response to SP and PWT2-SP (panel B). Concentration–response curve to SP (panel C) and PWT2-SP (panel D) in absence and in presence of aprepitant 10  $\mu$ M. Data are mean  $\pm$  SEM of four experiments performed in duplicate.

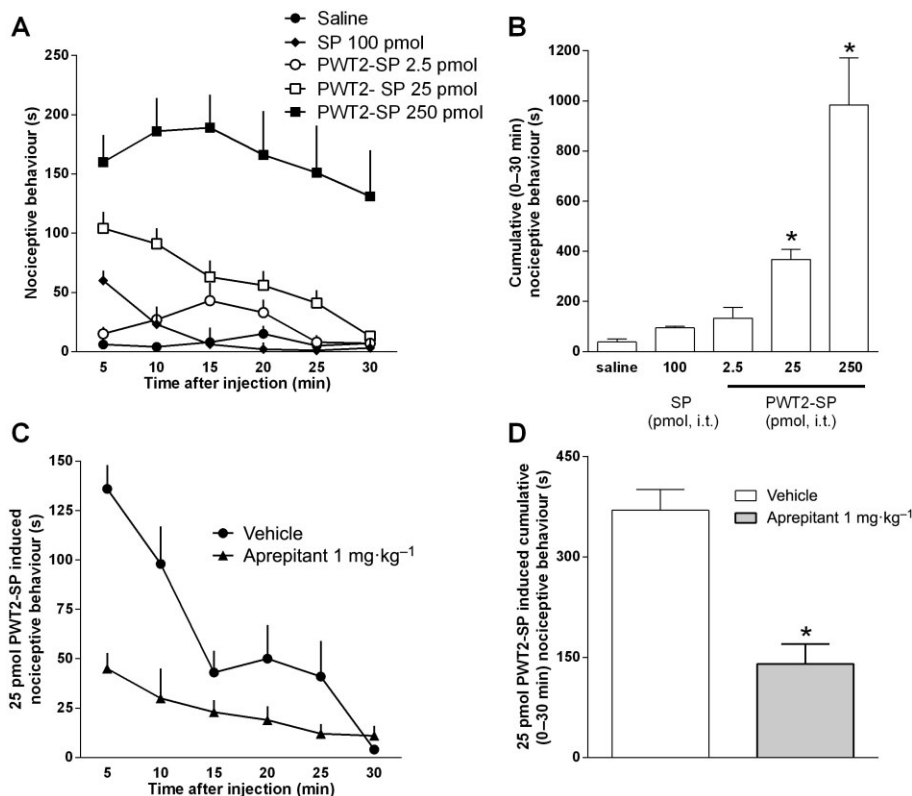
2014). Based on these findings, we can suggest that PWT technology used for generating these compounds has negligible impact on the ability of the peptide sequence to adopt the biologically active conformation responsible for full agonist activity.

With respect to selectivity of action, the following rank-order of potency has been measured for PWT2 tachykinins PWT2-SP > PWT2-NKA > PWT2-NKB, PWT2-NKA > PWT2-NKB > PWT2-SP, PWT2-NKB > PWT2-SP > PWT2-NKA at NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> receptors respectively. This rank-order (PWT2 tachykinins compared with natural tachykinins) demonstrated that PWT technology did not affect the selectivity of action of the natural peptide sequence. This finding is in line with previous results obtained with N/O/FQ PWT derivatives that maintain an extremely high selectivity of action over classical opioid receptors, typical of the natural peptide N/O/FQ (Rizzi *et al.*, 2014).

Considering potency, comparison of each PWT2 derivative with the native peptide sequence suggests differences depending on both the peptide sequence and the receptor under study. In particular, PWT2-NKB always displayed similar potency to NKB, and PWT derivatives displayed

similar potency to the natural peptides at NK<sub>3</sub> receptors. In contrast, PWT2-SP and PWT2-NKA displayed lower potency compared with SP and NKA at NK<sub>1</sub> and NK<sub>2</sub> receptors. We cannot explain those differences, but the present results indicate that agonist potency may or may not be affected by the application of PWT technology depending on the peptide sequence and the receptor interacting with the ligand. Independently of agonist potency, the interaction of PWT derivatives with NK receptors seems to mirror that of the natural sequences as the NK receptor antagonists (aprepitant, GR159897, SB222200) displayed inhibitor potencies against PWT2-SP, PWT2-NKA and PWT2-NKB, close to those obtained in earlier studies against SP, NKA and NKB (Rizzi *et al.*, 2012). In addition, aprepitant behaved as an insurmountable antagonist against both PWT2-SP and SP with similar potency. Collectively, calcium mobilization studies demonstrated that tachykinin PWT derivatives behave as full agonists at human recombinant NK receptors with a selectivity profile superimposable to that of the parent peptides.

We further characterized the effects of PWT2-SP *in vitro* for its ability to promote the interaction of the NK<sub>1</sub> receptor with G-protein in BRET experiments and to evoke contractions of



**Figure 8**

SBL test in mice. Time course of the behaviour response to i.t. SP (100 pmol) and PWT2-SP (2.5–250 pmol, panel A); the same results are shown as cumulative nociceptive behaviour displayed over the 30 min period in the panel B. One-way ANOVA followed by the Dunnett's *post hoc* test revealed a significant effect of PWT2-SP [ $F_{(3,31)} = 19.87$ ]. Effects of pretreatment with aprepitant (1 mg·kg<sup>-1</sup>, for 2 h) on nociceptive behaviour induced by PWT2-SP (25 pmol). The time-course is shown in panel C while the same results are shown as cumulative nociceptive behaviour displayed over the 30 min period in the panel D. Data are mean  $\pm$  SEM of at least eight mice per group. \* $P < 0.05$ , significantly different from saline or vehicle; unpaired *t*-test ( $t = 5.33$ , d.f. = 14).

the guinea pig ileum and rat urinary bladder. In the BRET assay SP produced a concentration-dependent stimulation of NK<sub>1</sub> receptor–G-protein association with high potency. PWT2-SP mimicked the action of SP and no differences were found between SP and PWT2-SP potency in this assay. To evaluate the action of PWT2-SP at native receptors the guinea pig ileum and rat urinary bladder were used. Guinea pig ileum studies were performed in the presence of atropine in order to block the effects of prejunctional NK<sub>3</sub> receptors, whose activation elicits ACh release (Laufer *et al.*, 1985). Under these experimental conditions, SP produced a concentration-dependent contraction with potency and maximal effects similar to those obtained in previous studies (Rizzi *et al.*, 2012). The effects of SP were mimicked by PWT2-SP with a concentration–response curve identical to that of the naturally occurring peptide. Experiments with the selective NK<sub>1</sub> receptor antagonist aprepitant demonstrated that the biological effects of PWT2-SP (and SP) are solely due to activation of NK<sub>1</sub> receptors. Very similar results have been obtained in the rat urinary bladder. The studies were performed in the presence of 1  $\mu$ M SR 48968 to exclude the interference of the NK<sub>2</sub> receptor (Meini and Maggi, 2010). Under these experimental conditions, SP produced a concentration-dependent contraction with potency and

maximal effects similar to those reported by Meini and Maggi (2010). The contractile action of SP was mimicked by PWT2-SP with similar potency and maximal effects. The exclusive involvement of the NK<sub>1</sub> receptor in the contractile effect of both SP and PWT2-SP was demonstrated by aprepitant sensitivity. Of note, the pharmacological features of aprepitant are very different at the rat NK<sub>1</sub> receptor (low potency, competitive behaviour) compared with the guinea pig or human receptor isoforms (very high potency, insurmountable behaviour). Identical results were previously obtained by us (Rizzi *et al.*, 2012) and others (Leffler *et al.*, 2009) investigating the effects of aprepitant at the rat and human recombinant NK<sub>1</sub> receptors. The reader is referred to Rizzi *et al.* (2012) for a detailed discussion of the mechanisms underlying the insurmountable antagonist behaviour of aprepitant. Collectively, bioassay studies at native receptors confirmed that PWT technology did not affect the pharmacological activity, high potency and selectivity of action of the natural peptide SP.

Interestingly, the potency of PWT2-SP at NK<sub>1</sub> receptors in BRET and bioassay experiments was identical to that of SP while, in calcium mobilization studies, PWT2-SP displayed an almost 30-fold lower potency. Previous studies demonstrated that PWT derivatives of N/OFQ displayed a reduced potency

in calcium mobilization studies while being more potent than the parent peptide in [<sup>35</sup>S]GTPγS binding and bioassay studies (Rizzi *et al.*, 2014). In isolated tissues, PWT-N/OFQ derivatives displayed slow onset of action and their effects were resistant to washing, while the action of N/OFQ occurred immediately after adding the peptide to the bath and was immediately reversible after washing. Thus, the relatively long time needed to obtain full activation of the N/OFQ receptor by PWT-N/OFQ might not be compatible with the rapid and transient nature of the calcium response. Thus, according to Charlton and Vauquelin (2010), we suggest that the reversal of the rank-order of agonist potency measured in the calcium assay compared with [<sup>35</sup>S]GTPγS binding and bioassay studies may derive from kinetic artefacts because of the hemiequilibrium conditions that characterize the calcium assay. The present bioassay studies, particularly the rat urinary bladder, also demonstrated kinetic differences between PWT2-SP and SP, namely slower onset of action and lower sensitivity to wash for PWT2-SP. Thus, similar kinetic artefacts may account for the discrepant results we obtained with PWT2-SP in the calcium mobilization studies where SP > PWT2-SP and in BRET and bioassay studies, where SP = PWT2-SP.

Collectively the *in vitro* studies demonstrated that the PWT technology can be applied to tachykinins for the generation of potent full NK receptor agonists that maintain a selectivity of action typical of the parent peptides. Based on these encouraging data, we further characterized the effects of PWT2-SP *in vivo*.

Previous experiments performed in our laboratories (Rizzi *et al.*, 2012) confirmed that i.t. injection of SP in mice elicited the characteristic SBL response. The action of SP was dose-dependent (10–1000 pmol) and short lasting. This was confirmed in the present experiments with 100 pmol SP. PWT2-SP in the dose range 2.5–25 pmol mimicked the effects of the natural peptide. However, PWT2-SP was more potent, produced more intense and longer-lasting nociceptive effects compared with the natural peptide. These results are similar to those obtained with N/OFQ and PWT-N/OFQ. Indeed, after supraspinal injection, in mice PWT-N/OFQ inhibited locomotor activity similar to N/OFQ; however, PWT-N/OFQ was more potent, produced more pronounced and longer-lasting effects (Rizzi *et al.*, 2014). Thus, high *in vivo* potency and long-lasting actions seem to be a common feature of PWT derivatives. These features could derive from the lower susceptibility to cleavage by peptidases, reported for multibranching peptides compared with free peptide sequences. This has been demonstrated for different peptide sequences including enkephalins, neurotensin and N/OFQ (Bracci *et al.*, 2003). A structure-based hypothesis of branched peptide resistance to proteolysis has been proposed by the same group (Falciani *et al.*, 2007). Peptide metabolism is likely to be more relevant *in vivo* than *in vitro*. Thus, it is reasonable to propose that the increase in agonist potency and duration of action displayed by multibranching peptides *in vivo* is largely due to their lower susceptibility to peptidase action. Another *in vitro* feature common to PWT derivatives of N/OFQ and SP is reduced sensitivity to washing, in organ bath experiments. This suggests longer-lasting target binding compared with native peptides. Several mechanisms have been proposed to explain the mode of action of multivalent ligands such as

PWT peptides. Receptor clustering, cooperative binding, rebinding and subsite binding (Gestwicki *et al.*, 2002) may be relevant. Longer lasting target binding might be important for the prolongation of *in vivo* drug action (for a detailed analysis of this topic see Vauquelin and Charlton, 2010). Thus different mechanisms (lower susceptibility to peptidases and longer-lasting target binding) alone or in combination may explain the high potency and long-lasting effects displayed by PWT derivatives *in vivo*.

Finally, the effects of PWT2-SP in the mouse SBL assay were sensitive to the NK<sub>1</sub> receptor antagonist, aprepitant, demonstrating the exclusive involvement of spinal NK<sub>1</sub> receptors. Identical results have been obtained previously by using this antagonist against the natural peptide SP (Rizzi *et al.*, 2012). These results demonstrate that while the PWT modification does increase potency and duration of action, it does so without affecting peptide selectivity *in vivo*.

In conclusion, the present study has shown that PWT technology can be successfully applied to the tachykinin peptide sequence to generate tetrabranching derivatives with an *in vitro* pharmacological profile similar to the native peptides. In addition, PWT2-SP demonstrated higher *in vivo* potency and a marked prolongation of action compared with SP. This NK<sub>1</sub> receptor-preferring ligand is therefore proposed as a novel research tool particularly useful to investigate those conditions in which a prolonged activation of the NK<sub>1</sub> receptor is desirable. More generally, this study confirms and extends previous findings demonstrating that the PWT strategy may be easily used to generate innovative and interesting ligands for peptidergic GPCRs.

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## Author contributions

CR and AR performed the *in vivo* experiments; CR, DM, MCC, FF, CA and CG performed the *in vitro* experiments; EM, RG and SS synthesized and purified the tachykinin tetrabranching derivatives; and GC wrote the first draft of the paper that has been finalized with the contributions of CR, TC and RG.

## Conflicts of interest

The following authors Girolamo Calo', Severo Salvadori, and Remo Guerrini are inventors of the patent application (EP13162532.9) focused on PWT technology and are founders of the University of Ferrara spin off company UFPeptides s.r.l. the assignee of such patent application.

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