Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 549 (2006) 140–148
www.elsevier.com/locate/ejphar

MEN15596, a novel nonpeptide tachykinin NK_2 receptor antagonist

Cecilia Cialdai [a], Manuela Tramontana [a], Riccardo Patacchini [a], Alessandro Lecci [a], Claudio Catalani [a], Rose-Marie Catalioto [a], Stefania Meini [a], Claudio Valenti [a], Maria Altamura [b], Sandro Giuliani [a,*], Carlo Alberto Maggi [a]

[a] *Pharmacology Department, Menarini Ricerche S.p.A, via Rismondo 12A, I-50131, Florence, Italy*
[b] *Chemistry Department, Menarini Ricerche S.p.A, via Rismondo 12A, I-50131, Florence, Italy*

Received 7 June 2006; received in revised form 31 July 2006; accepted 18 August 2006
Available online 26 August 2006

Abstract

The pharmacological profile of MEN15596 or (6-methyl-benzo[*b*]thiophene-2-carboxylic acid [1-(2-phenyl-1*R*-{[1-(tetrahydropyran-4-ylmethyl)-piperidin-4-ylmethyl]-carbamoyl}-ethylcarbamoyl)-cyclopentyl]-amide), a novel potent and selective tachykinin NK_2 receptor antagonist endowed with oral activity, is described. At the human recombinant tachykinin NK_2 receptor, MEN15596 showed subnanomolar affinity (pK_i 10.1) and potently antagonized (pK_B 9.1) the neurokinin A-induced intracellular calcium release. MEN15596 selectivity for the tachykinin NK_2 receptor was assessed by binding studies at the recombinant tachykinin NK_1 (pK_i 6.1) and NK_3 (pK_i 6.4) receptors, and at a number of 34 molecular targets including receptors, transporters and ion channels. In isolated smooth muscle preparations MEN15596 showed a marked species selectivity at the tachykinin NK_2 receptor with the highest antagonist potency in guinea-pig colon, human and pig bladder (pK_B 9.3, 9.2 and 8.8, respectively) whereas it was three orders of magnitude less potent in the rat and mouse urinary bladder (pK_B 6.3 and 5.8, respectively). In agreement with binding experiments, MEN15596 showed low potency in blocking selective NK_1 or NK_3 receptor agonist-induced contractions of guinea-pig ileum preparations ($pA_2 \leq 6$). In anaesthetized guinea-pigs, MEN15596 inhibited in a dose-related and persistent manner colon contractions induced by the selective tachykinin NK_2 receptor agonist, [βAla^8]neurokinin A(4–10) (3 nmol/kg i.v.), either after intravenous (ED_{50} 0.18 μmol/kg), intraduodenal (ED_{50} 3.16 μmol/kg) or oral administration (10–30 μmol/kg) without affecting, at 3 μmol/kg, i.v., the colonic contractions produced by the NK_1 receptor selective agonist [Sar^9]substance P sulfone (3 nmol/kg i.v.). In addition MEN15596 was effective in inhibiting bronchoconstriction produced by i.v. administration of [βAla^8]neurokinin A(4–10). Overall the results indicate that MEN15596 is a potent and selective tachykinin NK_2 receptor antagonist possessing high affinity and potency for guinea-pig, pig and human receptor, long duration of action in *in vivo* experiments and good oral bioavailability.

Keywords: Tachykinins; Neurokinin A; NK_2 receptor antagonist; Oral activity; Colon contraction; Gastrointestinal disorders; Bronchoconstriction

1. Introduction

Tachykinins are a family of peptides widely expressed in the central nervous system and at the periphery, in both neurons and other kinds of cells. Three receptors, termed NK_1, NK_2, and NK_3 mediate the effects of tachykinins. Although all tachykinins can act as full agonists at the three receptors, substance P and hemokinin-1 preferentially activate NK_1 receptors, whereas neurokinin A and B are preferential agonists at NK_2 and NK_3 receptors, respectively (Patacchini et al., 2004).

Tachykinin NK_2 receptors are widely expressed in peripheral mammal tissues, especially at the visceral level. Tachykinin NK_2 receptor agonists induce contraction of human airways, urinary bladder and gastrointestinal smooth muscle (Giuliani et al., 1991, 1993; Astolfi et al., 1994; Patacchini et al., 2000). In animal models of airway diseases the administration of selective NK_2 receptor antagonists reduce bronchial hyperreactivity and the number of inflammatory cells in the bronchoalveolar fluid, highlighting a NK_2 receptor-mediated component of cellular inflammation (Schuiling et al., 1999; Maghni et al., 2000; Tramontana et al., 2002). Furthermore, other studies indicate that tachykinins acting through NK_2 receptors can induce visceral hyperalgesia in urinary bladder (Kiss et al., 2001) and

* Corresponding author. Tel.: +39 055 5680750; fax: +39 055 5680419.
E-mail address: sgiuliani@menarini-ricerche.it (S. Giuliani).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.08.021

gastrointestinal tract (Lecci et al., 2004) possibly through a direct effect on sensory neurons (Sculptoreanu and de Groat, 2003). In particular, in the gastrointestinal tract, beyond visceral hyperalgesia, tachykinin NK_2 receptor antagonists also reduced hypermotility and hypersecretions in animal models of gastrointestinal diseases without altering these functions under control conditions. These results suggest that selective antagonists possessing good oral bioavailability could represent a valid option for the pharmacological treatment of diarrhea-prevalent irritable bowel syndrome (Lecci et al., 2004). Nepadutant, a bicyclic hexapeptide with potent and selective antagonist activity at NK_2 receptor, has been shown to possess an interesting profile in human models of altered intestinal motility and perception (Lordal et al., 2001), however the limited oral bioavailability of this drug has hindered further clinical development for irritable bowel syndrome and prompted the search for an orally active NK_2 receptor antagonist as drug candidate for the treatment of intestinal disorders. MEN15596 or (6-methyl-benzo[*b*]thiophene-2-carboxylic acid [1-(2-phenyl-1*R*-{[1-(tetrahydropyran-4-ylmethyl)-piperidin-4-ylmethyl]-carbamoyl}-ethylcarbamoyl)-cyclopentyl]-amide) (Fig. 1) is a small, nonpeptide molecule, rationally designed in our laboratories to achieve both antagonist potency and oral bioavailability, and at the same time, to maintain a low level of complexity in the chemical structure.

This study aims to provide the *in vitro* and *in vivo* pharmacological characterization of MEN15596. In particular, its affinity and selectivity for the recombinant human NK_2 receptor expressed on CHO cells was estimated by means of binding and functional experiments. Binding studies were also used to evaluate the affinity of MEN15596 at more than 30 different receptors, transporters and ion channels. The antagonist potency of MEN15596 was assessed at the recombinant and native human NK_2 receptor. Moreover, a species selectivity was determined through functional studies on isolated organs. Finally, MEN15596 *in vivo* inhibitory potency and duration of effect following i.v., i.d. or oral route of administration was assessed on NK_2 receptor agonist-induced colonic contractions and bronchoconstriction in anaesthetized guinea-pigs.

2. Materials and methods

2.1. CHO cells membrane preparation

CHO-K1 cells stably expressing the human NK_1 receptor, prepared in Menarini Ricerche (Pomezia, Italy) or the human NK_2 receptor, kindly provided by Dr. J.E. Krause (Washington University, MO, USA) were cultured in F12 and α-modification

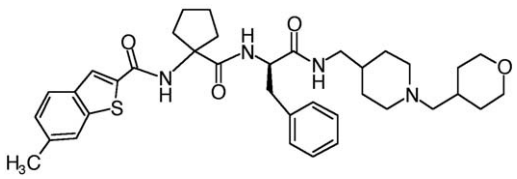

Fig. 1. Chemical structure of MEN15596 or 6-methyl-benzo[*b*]thiophene-2-carboxylic acid [1-(2-phenyl-1*R*-{[1-(tetrahydropyran-4-ylmethyl)-piperidin-4-ylmethyl]-carbamoyl}-ethylcarbamoyl)-cyclopentyl]-amide.

essential Eagle's medium (α-MEM), respectively, supplemented with 2 mM L-glutamine and foetal bovine serum (FBS, 10%). Cells were subcultured by using 0.25% trypsin and 1 mM ethylenediaminetetraacetate (EDTA) to detach them and then cultured in 175 cm^2 flasks in a humidified atmosphere at 37 °C with 5% CO_2.

Cells at confluence were washed out of the medium by Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, harvested and washed in trishydroxymethyl aminomethane buffer (TRIS, 50 mM, pH 7.4, at 4 °C) containing thiorphan 10 μM, leupeptin 5 μg/ml, bacitracin 100 μg/ml and chymostatin 10 μg/ml and homogenized with a Polytron (PT 3000, Kinematica), set at 15,000 rpm for 30 s. The homogenate was centrifuged at 45,000 g for 45 min (4 °C). The membrane pellet was resuspended in TRIS buffer to obtain 5 mg/ml protein concentration and frozen immediately in 1 ml aliquots by immersion in liquid nitrogen, and then stored at −80 °C until use.

The protein concentration was determined by the method of Bradford (1976) with a Bio-Rad kit, using bovine serum albumin as reference standard. Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

2.2. Radioligand binding

The buffer used for binding experiments was TRIS (50 mM, pH 7.4) containing the described enzyme inhibitors, $MnCl_2$ 5 mM, and bovine serum albumin (1 g/l). Binding assay was performed at room temperature in a final volume of 0.5 ml and with a different incubation time according to the radioligand used and receptor assay: 30 min for [^{125}I]neurokinin A at the human tachykinin NK_2 receptor and 60 min for [^{3}H]substance P at the human tachykinin NK_1 receptor. Each radioligand was used at a concentration less than 10% of the total added radioligand concentration giving a specific binding which represented approximately the 70–80% of the total binding. Non-specific binding was defined as the amount of radiolabelled ligand bound in the presence of the appropriate unlabelled ligand (1 μM). Competing ligands were tested in a wide range of concentrations (1 pM–1 μM). The final dimethylsulphoxide (DMSO) concentration in the assay was 1% and it did not affect radioligand binding.

All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard) that have been pre-soaked for at least 2 h in polyethylenimine 0.3%, using a MicroMate 96 Cell Harvester (Packard Instrument Company). Tubes and filters were then washed 5 times with 0.5 ml aliquots of TRIS buffer (50 mM, pH 7.4, 4 °C). Filters were dried and soaked in Microscint 40 (Packard Instrument Company) and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company). Each experiment was performed in duplicate. Furthermore the binding affinity of MEN15596 at a range of 34 different receptors, including the human tachykinin NK_3 receptor, neurotransmitter transporter and ion channels (see Results) was measured according to established methods at Cerep (le Bois l'Eveque BP, 186600 Celle l'Evescault, France).

2.3. Intracellular calcium transient measurements

Cytosolic free calcium ($[Ca^{2+}]_i$) was measured in cell suspensions using the Ca^{2+} fluorescent dye, Fura-2. Confluent cells were washed twice with Dulbecco's phosphate buffer saline without Ca^{2+} and Mg^{2+} and harvested by treatment with a non-enzymatic cell dissociation solution (Sigma). After harvesting and centrifugation at 500 ×*g* for 10 min, the cells were incubated in oxygenated Krebs–Henseleit solution (pH 7.4) having the following composition in mM: NaCl 119; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4$ 1.5; KCl 4.7; $CaCl_2$ 2.5 and glucose 11) supplemented with 0.1% BSA, and 5 μM Fura-2/AM for 30 min at 37 °C with occasional shaking.

Thereafter the cells were washed with Krebs-buffer saline, plus 0.5% BSA and 0.5 mM probenecid, and the incubation was prolonged for 35 min at room temperature to allow complete hydrolysis of the dye by the intracellular esterases. The Fura-2 loaded cells were washed again and finally resuspended in Krebs-buffer plus 0.5 mM probenecid for immediate measurement of Ca^{2+}. For measurements, cells were transferred in a water-jacketed thermostated cuvette (37 °C) with continuous stirring and fluorescence was monitored at an emission wavelength of 509 nm using a Perkin–Elmer LS-50B spectrofluorimeter with excitation wavelengths switching continuously between 340 and 380 nm. The obtained fluorescence intensities were analysed by the customised software Winlab provided by Perkin–Elmer. The changes of the ratio of Fura-2 fluorescence intensities, ΔF340/F380, obtained after subtraction of the respective background, were used as an index of the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. The values of $[Ca^{2+}]_i$ were calculated (Grynkiewicz et al., 1985) as

$$[Ca^{2+}]_i = K_D \times (R - Rmin)/(Rmax - R) \times \beta, \quad (1)$$

where K_D is the dissociation constant for Fura-2 (224 nM); Rmax and Rmin are the ratios for bound and unbound forms of Fura-2, respectively; and β is the ratio between maximum and minimum fluorescence intensities of Fura-2 at 380 nm excitation. For the calibration procedure, maximum and minimum fluorescence signals were obtained by adding ionomycin (10 μM) and EGTA (6.25 mM) sequentially at the end of the experiment. The autofluorescence value was less than 15% of the Fura-2 signals at either excitation wavelengths.

2.4. Isolated organs functional studies

Male albino New Zealand rabbits (2.5–3.0 kg, Charles River, Calco, Italy), male Dunkin–Hartley guinea-pigs (250–300 g, Charles River, Calco, Italy), male Wistar rats (300–350 g, Harlan Italy, Udine, Italy), male Swiss CD-1 mice (25–30 g, Harlan Italy, Udine, Italy) and minipigs (7–10 kg, Ellegaard Gottingen Minipigs, Dalmose, Denmark) were sacrificed following the principle and guidelines of the European Union regulation and local ethical committee approval. Guinea-pig proximal colon circular muscle strips, guinea-pig ileum longitudinal muscle myenteric plexus strips, rabbit pulmonary artery circular muscle strips deprived of the endothelium and rat, mouse, pig and human urinary bladder detrusor muscle strips, were prepared for recording mechanical responses to tachykinin receptor agonists. Human urinary bladder muscle strips were obtained from 6 patients (age 48–75, both sexes), undergoing cystectomy for bladder base carcinoma (see Meini et al., 2000 for details). For human and pig urinary bladder, strips of 2 cm length have been prepared and stored overnight in oxygenated Krebs–Henseleit solution at 4 °C to be used the following day.

All preparations were placed in 5 ml organ baths filled with oxygenated (96% O_2 and 4% CO_2) Krebs–Henseleit solution. The contractile activity of guinea-pig colon (load 10 mN), guinea-pig ileum (load 3 mN), rat (load 5 mN) and mouse urinary bladder (load 5 mN) was recorded isotonically, while that of human and pig urinary bladder (load 10 mN) and rabbit pulmonary artery was recorded isometrically.

The activity of MEN15596 at tachykinin NK_2 receptors of guinea-pig colon was tested against the selective NK_2 receptor agonist [βAla^8]neurokinin A(4–10) in the presence of the NK_1 receptor-selective antagonist SR140333 (Emonds-Alt et al., 1993). The activity in rabbit pulmonary artery, mouse, pig and human urinary bladder was evaluated against neurokinin A contractile response, that in rat urinary bladder was studied by using [βAla^8]neurokinin A(4–10) as selective agonist in the presence of indomethacin (10 μM).

The activity of MEN15596 at tachykinin NK_1 and NK_3 (Patacchini et al., 1995) receptors was checked in guinea-pig ileum in the presence of chlorpheniramine (1 μM) and indomethacin (10 μM), with the addition of atropine (1 μM) for NK_1 receptor study and SR140333 (0.1 μM) for the NK_3 receptor activity test. As NK_1 and NK_3 selective tachykinin receptor agonists substance P methyl ester (SPOMe) and senktide were used, respectively.

All the experiments in pig urinary bladder have been performed in the presence of SR140333 (0.3 μM) and SR142801 (0.3 μM) (Emonds-Alt et al., 1995; Patacchini et al., 1995) to exclude the involvement of the tachykinin NK_1 and NK_3 receptor in the neurokinin A-induced responses.

After 1 h stabilization period the preparations were repeatedly contracted to stabilize the tissue responsiveness with KCl 80 mM every 30 min for 3–4 times, except the rabbit pulmonary artery where neurokinin A (1 μM) was used.

In experiments aiming to calculate the affinity of MEN15596, two cumulative concentration–response curves to the agonists were obtained, each concentration being added when the effect of the preceding one has reached a steady state: the first concentration–response curve in the absence (control curve) and the second in the presence of MEN15596 after a contact period of 15–30 min.

Only one concentration of antagonist was tested in each preparation.

2.5. In vivo experiments

All the experiments were carried out in accordance with the Declaration of Helsinki, with the principles and guidelines of the European Union regulations and the local ethical committee.

Male albino guinea-pigs (Charles River, Italy) weighing 350–400 g were fasted 24 h before experiments with free access to water.

The animals were anaesthetized with urethane (1.5 g/kg, s.c.) and a polyethylene catheter was inserted into the left jugular vein for drugs administration.

Guinea-pigs were mechanically ventilated through a tracheal cannula connected to a ventilation pump (Basile mod. 7025) adjusted at a rate of 50 strokes/min and respiration volume of 10 ml/kg. The body temperature was kept constant at 36 °C by a thermoregulated lamp.

The abdomen was opened and a latex balloon, constituted by a condom head, connected to a PE90 polyethylene catheter, was inserted into the proximal colon at about 2–3 cm from the caecum and filled with 0.5 ml of saline. The intracolonic balloon was connected to a pressure transducer (Transpac IV, Abbott, Italy) for intraluminal pressure recording by means of a MacLab/8S ML 780 data acquisition system (ADInstruments, UK).

5 min before starting the experiments, guinea-pigs were treated with hexamethonium bromide (13.8 μmol/kg, i.v.) as bolus followed by continuous infusion of the same solution at the rate of 300 μl/h to prevent reflex cholinergic responses. MEN15596 or its vehicle (DMSO) were administered i.v. (0.3–3 μmol/kg) or i.d. (1–10 μmol/kg) in a volume of 100 μl/kg or 1 ml/kg, respectively.

[βAla^8]neurokinin A(4–10) was administered 2 or 3 times before the antagonist or the vehicle administration in order to stabilize the contractile responses and the challenge repeated at 5, 30 and then every 30 min until 4 h after antagonist administration.

The dose of agonist for time-course experiments was selected on the basis of dose–response experiments where [βAla^8] neurokinin A(4–10) was administered at increasing doses in the same animal at 30 min intervals in the experimental conditions as described above. The selected dose of [βAla^8]neurokinin A (4–10) (3 nmol/kg, i.v.) corresponds approximately to the ED_{50}, in terms of maximal amplitude of colonic contractions.

In order to evaluate the activity of MEN15596 following its oral administration, the compound or its vehicle (0.5% carboxymethylcellulose, 0.4% Tween 80 in distilled water) was administered by gavage (5 ml/kg) in fasted unanaesthetized guinea-pigs. Animals were anaesthetized 45 min later with urethane (1.5 g/kg sc). The [βAla^8]neurokinin A(4–10) challenge (3 nmol/kg i.v.) was performed 3.5 h after antagonist or the vehicle administration and then every 30 min until 9 h in hexamethonium-pretreated animals (see above).

In another series of experiments the pulmonary insufflation pressure was measured by attaching a side arm of the tracheal cannula to a pressure transducer (Transpac IV, Abbott, Italy) and recorded by means of a MacLab/8S ML 780 data acquisition system (ADInstruments, UK).

The selectivity of MEN15596 (3 μmol/kg, i.v., 5 min before) was tested against the colonic contraction induced by i.v. administration of the selective tachykinin NK_1 receptor agonist [Sar^9]substance P sulphone (3 nmol/kg, i.v.) and then (30 min later) compared with the response to [βAla^8]neurokinin A (4–10) (3 nmol/kg, i.v.).

The colonic contractions and bronchoconstriction were calculated as amplitude (mm Hg). The effect of MEN15596 (i.v. or intraduodenal, i.d., administration) at various time-points was expressed as percentage of inhibition of the basal response (before its administration) or as percentage of inhibition of the averaged control response of vehicle-treated animals (oral administration).

2.6. Statistical analysis

All values in the text, tables or figures are mean and 95% confidence limits (95% c.l.), or ± S.E.M. of the given (n) number of experiments. Statistical analysis was performed by means of Student's t-test for paired or unpaired data or by means of two-way analysis of variance (ANOVA) followed by Fisher's least significance tests. A P level < 0.05 was considered statistically significant.

Inhibition curves from binding experiments were analysed by fitting the data with GraphPad Prism 4.0 program (San Diego, CA) to determine the radioligand affinity constant (K_d) and the IC_{50} values in order to calculate the equilibrium inhibition constant (K_i).

The apparent affinity of the antagonists was expressed in terms of apparent pK_B (negative logarithm of the antagonist dissociation constant) and, assuming a slope of −1, it was estimated by the equation: $pK_B = \log [\text{dose ratio} - 1] - \log [\text{antagonist concentration}]$ (Kenakin, 1997; Jenkinson, 1991).

The competitive nature of MEN15596 antagonism was checked by Schild plot analysis (Arunlakshana and Schild, 1959).

2.7. Drugs

MEN15596 or 6-methyl-benzo[b]thiophene-2-carboxylic acid[1-(2-phenyl-1R-{[1-(tetrahydropyran-4-ylmethyl)-piperidin-4-ylmethyl]-carbamoyl}-ethylcarbamoyl)-cyclopentyl]-amide was synthesized at the Chemistry Department of Menarini Ricerche (Florence and Pomezia, Italy).

[^{3}H]-substance P (specific activity 41 Ci·mmol^{-1}) and [^{125}I]-neurokinin A (specific activity 2000 Ci·mmol^{-1}) were provided by Amersham International (Buckinghamshire, U. K.). Leupeptin was obtained from Boehringer Mannheim (Germany).

Neurokinin A and [βAla^8]neurokinin A(4–10) were from EspiKem (Florence, Italy). Thiorphan, substance P, senktide, SPOMe and [Sar^9]substance P sulfone were purchased from Bachem (Bubendorf, Switzerland). Other drugs used were: bacitracin, chymostatin, indomethacin, chlorpheniramine maleate, hexamethonium bromide (Sigma, St. Louis, MO, USA); atropine sulfate (Serva, Heidelberg, Germany); Fura2 was from Calbiochem (Schwalbach, Germany).

The nonpeptide antagonists SR140333, [(*S*)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl] ethyl}-4-phenyl-1-azoniabicyclo [2,2,2] octane chloride] and SR142801, ((*S*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl) piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-*N*-methylacetamide) were a kind gift of Dr. X. Emonds-Alt (Sanofi Recherche, Montpellier, France).

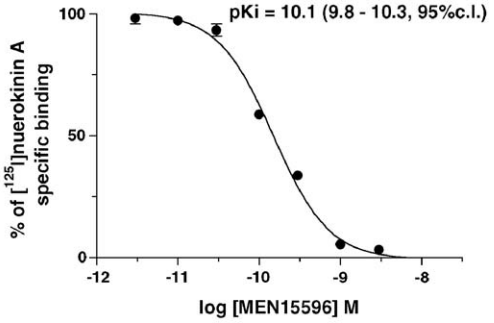

Fig. 2. MEN15596 concentration-dependently inhibits the [^{125}I]-neurokinin A binding at the human recombinant tachykinin NK_2 receptors stably expressed in CHO-K1 cells. Each value is the mean±S.E.M. of 3 experiments.

All salts used were purchased from Merck (Darmstadt, Germany).

Stock solutions of MEN15596 (10 mM) were prepared in dimethylsulfoxide (DMSO).

CHO-K1 cells, transfected with the human NK_2 receptor, were provided by Dr. J.E. Krause (Washington University, School of Medicine, St Louis, MO).

3. Results

3.1. Affinity and selectivity of MEN15596 for the human tachykinin NK_2 receptor

MEN15596 (3 pM–10 nM) concentration-dependently inhibited the [^{125}I]neurokinin A binding to membranes of CHO-K1 cells expressing the human tachykinin NK_2 receptor and the resulting affinity, measured as pK_i value, was 10.1 (9.8–10.3, 95% c.l.) (n=3, Fig. 2, Table 1), being 30-fold more potent than nepadutant, a tachykinin NK_2 receptor antagonist (Catalioto et al., 1998) with pK_i 8.6 (8.5–8.7, 95% c.l.). The tachykinin NK_2 receptor selectivity of MEN15596 was checked at the recombinant human NK_1 and NK_3 receptor (CHO cell membranes). MEN15596 inhibited the binding of the agonist, [^{3}H] substance P, and antagonist, [^{3}H]SR142801, radioligands to the human NK_1 and NK_3 receptor, respectively, with affinity values of 6.1 (6.0–6.3, 95% c.l.) and 6.2 (5.9–6.5, 95% c.l.) (Table 2), showing a potency, at these tachykinin receptor subtypes, four orders of magnitude lower as compared to the NK_2 one.

The *in vitro* selectivity profile of MEN15596 was evaluated in binding assays *vs.* 26 receptors (50% human), 5 ion channels and 3 transporters at the concentration of 10 μM (Table 3). In the large majority of these tests, MEN15596 showed a very low affinity, with inhibition of specific binding ≤50% at 10 μM concentration. In 3 cases out of 34 (non-selective muscarinic receptor, L-type calcium channel, veratridine-sensitive sodium channel) the percentage of inhibition at 10 μM was in the range 50–70%. For other 3 receptors (non-selective opiate, non-selective σ, vasopressin $hV1_\alpha$), with inhibition ≥70%, binding assays were further determined at different concentrations in order to calculate the pK_i values. In all these three cases, the affinity (pK_i 5.6–6.9) was over 1000-fold lower than that measured at the human NK_2 receptor.

3.2. Antagonist potency of MEN15596 at the human NK_2 tachykinin receptor

In CHO-K1 cells expressing the human tachykinin NK_2 receptor, neurokinin A (1 nM–10 μM) induced a concentration-dependent release of intracellular calcium with an EC_{50} of 7.6±1.7 nM. MEN15596 (10 nM, 15 min contact time) inhibited the neurokinin A-induced intracellular calcium release, with pK_B=9.1±0.05, (n=3) (Table 1) with about 5-fold more potency than nepadutant (pK_B=8.4±0.2, n=3).

MEN15596 behaved as an antagonist at subnanomolar concentrations, even at the native human NK_2 receptor. In the isolated human urinary bladder smooth muscle, neurokinin A produced concentration-dependent contractile responses which were antagonized by MEN15596 with a pK_B value of 9.2±0.2 (n=6), showing also in this test higher potency (6-fold) as compared to nepadutant (pK_B=8.5±0.2, n=5).

3.3. Functional tests in different animal species

In isolated guinea-pig colon, in the presence of the NK_1 receptor antagonist SR140333, MEN15596 competitively inhibited NK_2 receptor-mediated contractions at subnanomolar concentrations. Schild plot analysis showed a competitive antagonism with a pK_B=9.3±0.07 and slope 1.19 (0.9–1.4 95% c.l.) (n=13) (Fig. 3, Table 1). In the same test, nepadutant has shown a potency lower by about one order of magnitude (pK_B=8.1±0.07). The selectivity of MEN15596 towards the NK_2 receptor was evaluated also in the isolated guinea-pig ileum where it antagonized the contractions produced by the selective NK_1 agonist SPOMe with pK_B ≤5.0 (n=3). Furthermore the antagonist activity of MEN15596 at the NK_3 receptor in guinea-pig ileum vs. senktide as agonist (in the presence of SR140333, see methods) indicated a low affinity (pK_B=6.0±0.2,

Table 1
In vitro pharmacological characterization of MEN15596: affinity at tachykinin NK_2 receptor in binding and functional tests

Compound	hNK_2–CHO binding	hNK_2–CHO $[Ca^{2+}]_i$	Human bladder	Guinea pig colon	Minipig bladder	Rabbit pulmonary artery	Rat bladder	Mouse bladder
	pK_i	pK_B	pK_B	pK_B	pK_B	pK_B	pK_B	pK_B
MEN15596	10.1 (9.8–10.3 c.l.)	9.1±0.05	9.2±0.2	9.3±0.07	8.8±0.1	7.5±0.08	6.3±0.1	5.8±0.2
Nepadutant	8.6[a] (8.5–8.7 c.l.)	8.4±0.2	8.5±0.2	8.1±0.07[b]	nt	8.6±0.07[a]	9.0±0.04[a]	9.8±0.15[a]

Each value is the mean±S.E.M. of pK_i or pK_B (negative logarithm of the antagonist dissociation constant) obtained from 3–13 experiments. nt: not tested. In brackets 95% confidence limits.
[a]data from Catalioto et al., 1998; [b]data from Santicioli et al., 1997.

Table 2
Selectivity of MEN15596: *in vitro* activity *vs.* the tachykinin NK_1 and NK_3 receptors in CHO cells transfected with the human receptors and in guinea-pig isolated ileum

Compound	NK_1[^{3}H]SP human binding (pK_i)	NK_2 guinea-pig ileum (pK_B)	NK_3 [^{3}H] SR142801 human binding (pK_i)	NK_3 guinea-pig ileum (pK_B)
MEN15596	6.1 (6.0–6.3)	≤5.0	6.2 (5.9–6.5)	6.0±0.2
Nepadutant	<6	5.7±0.04[a]	<6	5.9±0.8[a]

Each value is the mean±S.E.M. of 4–8 experiments.
In guinea-pig isolated ileum tachykinin receptor selective agonists were SPOMe and senktide for NK_1 and NK_3 receptor, respectively.
In brackets 95% confidence limits.
[a]data from Catalioto et al., 1998.

n=4). Pig urinary bladder was another tissue where MEN15596 competitively and with high potency inhibited neurokinin A-induced contractions with pK_B=8.8±0.1 (n=12) and a Schild plot slope of 0.99 (0.94–1.04 95% c.l.).

In other functional tests in isolated animal tissues, MEN15596 was characterized by high species selectivity. In rabbit pulmonary artery it had one log unit lower affinity than nepadutant with pK_B=7.5±0.08 (n=5) *vs.* pK_B=8.6±0.07, respectively. In rat and mouse urinary bladder MEN15596 was active only at micromolar concentrations, with pK_B=6.3±0.1 in rat urinary bladder (n=4) *vs.* pK_B=9.0±0.04 for nepadutant, and pK_B=5.8±0.2 in mouse urinary bladder (n=4) *vs.* pK_B=9.8±0.15 for nepadutant (Table 1).

Table 3
Selectivity of MEN15596: binding activity at 10 μM concentration *vs.* various receptors, ion channels and transporters

Assay	% inhibition of specific binding
Human adenosine A_1 receptor	3
Human adenosine A_{2A} receptor	8
α_1 adrenergic receptor (non-selecteive)	40
α_2 adrenergic receptor (non-selective)	24
Human β_1 adrenergic receptor	−3
Human angiotensin AT_1 receptor	5
Benzodiazepine receptor (central)	9
Human bradykinin B_2 receptor	2
Human cholecystokinin CCK_A receptor	5
Human dopamine D_1 receptor	4
Human dopamine D_2 receptor	−1
Human endothelin ET_A receptor	8
GABA (non-selective)	0
NMDA	2
Histamine H_1 (central)	0
Human melanocortin MC_4 receptor	9
Muscarinic receptor (non-selective)	56
Neuropeptide Y receptor (non-selective)	−13
Nicotinic (neuronal)	2
Opiate (non-selective)	73(pK_i=5.6)
Human ORL 1 receptor (NOP)	1
Phencyclidine receptor	13
5-HT receptor (non-selective)	6
σ receptor (non-selective)	96(pK_i=6.9)
Human glucocorticoid receptor (GR)	−25
Human vasopressin V_{1a} receptor	95(pK_i=6.4)
Ca^{2+} channel (L-type, dihydropyridine site)	52
K^+ channel (K_V)	−3
K^+ channel (SK_{Ca})	6
Na^+ channel (site 2)	66
Cl^- channel	−3
Human norepinephrine transporter	5
Dopamine transporter	−12
5-HT transporter	9

In brackets the pK_i values from competition curves obtained with MEN15596 at the corresponding receptor.

3.4. Effect of MEN15596 on colon contractions induced by intravenous administration of [βAla^8]neurokinin A(4–10)

The selective tachykinin NK_2 receptor agonist [βAla^8]neurokinin A(4–10) (0.1–100 nmol/kg i.v., n=7) induced dose-dependent colon contractions in anaesthetized and hexamethonium-treated guinea-pigs. The dose of 3 nmol/kg, inducing about 40% of the maximal response, was selected for further studies with MEN15596 (data not shown). Intravenous administration of [βAla^8]neurokinin A(4–10) (3 nmol/kg) at 30 min intervals induced reproducible colon contractions averaging 11±3 mm Hg (n=20). In vehicle treated animals the amplitude of this response did not significantly change during the 4th observation period (Figs. 4A–B and 5). The intravenous administration of MEN15596 (0.3–3 μmol/kg) in anaesthetized guinea-pigs induced a dose-dependent inhibition of the colon contractions produced by [βAla^8]neurokinin A(4–

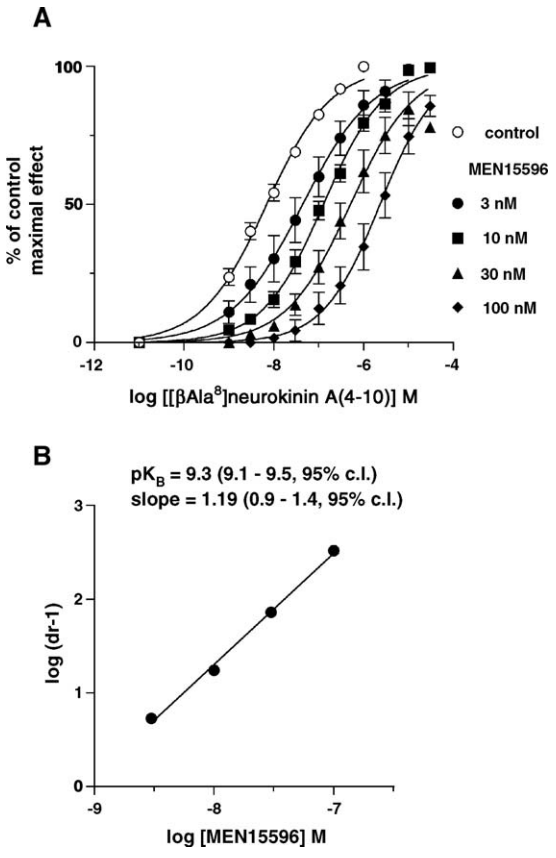

Fig. 3. Inhibitory effect of MEN15596 on contractions induced by tachykinin NK_2 receptor stimulation with the selective agonist [βAla^8]neurokinin A(4–10), in guinea-pig isolated colon. The experiments were performed in the presence of the tachykinin NK_1 receptor antagonist SR 140333 (1 μM) (panel A). Each value is the mean±S.E.M. of 13 experiments. The panel B shows the relative Schild plot of agonist dose ratios vs. MEN15596 concentrations (slope=1.19, 0.9–1.4 95% confidence limits).

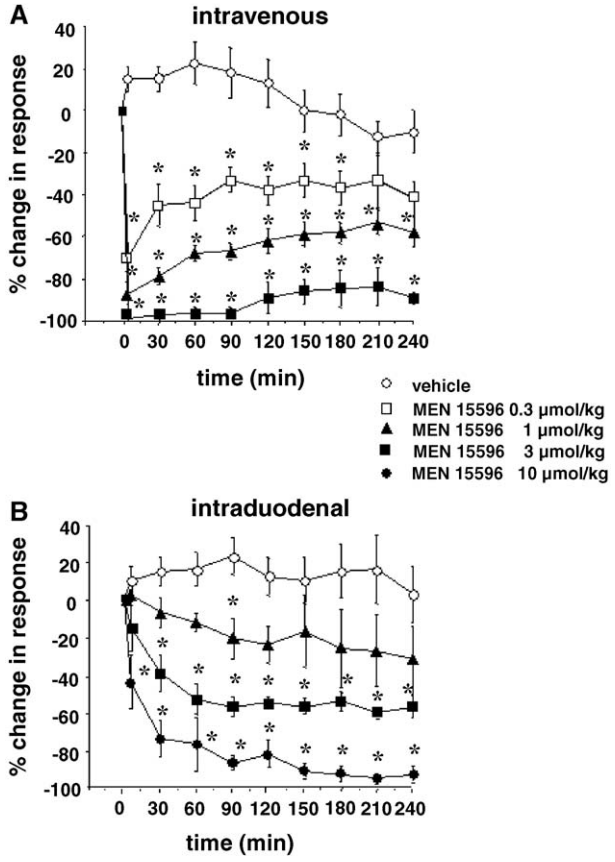

Fig. 4. Inhibitory effect of intravenous (A) or intraduodenal (B) administration of MEN15596 on colon contractions induced by intravenous administration of [βAla^8]neurokinin A(4–10) (3 nmol/kg) in anaesthetized guinea-pigs. In all experiments the animals were treated with hexamethonium bromide as a bolus (13.8 µmol/kg, i.v.) followed by continuous infusion of the same solution (300 µl/h) for the duration of the experiment. The results are expressed as % change of the control response to [βAla^8]neurokinin A-(4–10) before the treatment. Each value is the mean±S.E.M. of 4–20 experiments. *, $P<0.05$, significantly different from the corresponding value in the vehicle group.

10) (3 nmol/kg, i.v.) (Fig. 4A). The maximal inhibitory effect was reached rapidly within 5 min from the NK_2 antagonist administration (70±7%; 87±5%; 99±1% inhibition at 0.3, 1 and 3 µmol/kg, respectively) and the response to the agonist slowly recovered thereafter but was still depressed up to 240 min. The ED_{50} after intravenous administration was 0.18 µmol/kg (0.14–0.22, 95% c.l.).

After i.d. administration, MEN15596 (1–10 µmol/kg) produced a dose-dependent inhibition of [βAla^8]neurokinin A (4–10)-induced colon contractions in guinea-pigs (Fig. 4B). The effect was slower in onset than after i.v. administration but when the maximum effect was reached it remained stable up to 4 h at all doses tested. Significant inhibitory effects were observed from 1 µmol/kg. At the highest dose (10 µmol/kg), MEN15596 inhibited by about 75% the control response and this effect was increased thereafter until reaching about 95% at 4 h (Fig. 4B). The ED_{50} after intraduodenal administration was 3.16 µmol/kg (2.41–4.13, 95% c.l.).

Oral administration of MEN15596 (10–30 µmol/kg) produced a dose-dependent inhibitory effect on colon contractions induced by the selective NK_2 agonist (3 nmol/kg i.v.) (Fig. 5) for the whole experimental period after 3.5 to 9 h from antagonist administration. The inhibition at 10 µmol/kg was about 30–40% and peaked at 6 h (49±7% inhibition, $n=6$). At the highest dose (30 µmol/kg, os), MEN15596 showed a maximal inhibitory effect at 4 h (82±9%, $n=8$) from its administration and the contraction to the agonist slowly recovered thereafter reaching 40–50% inhibition, after 9 h from antagonist administration.

3.5. Effect of MEN15596 on bronchoconstriction induced by intravenous administration of [βAla8]neurokinin A(4–10)

The intravenous administration of [βAla^8]neurokinin A(4–10), at 3 nmol/kg i.v., induced a reproducible increase of respiratory insufflation pressure at 30 min interval averaging 34±3 mm Hg ($n=12$) in anaesthetized guinea-pigs. In vehicle treated animals the amplitude of this response did not show significant time-dependent changes over 4 h observation period.

MEN15596 (1–3 µmol/kg, Fig. 6) produced a dose-dependent inhibition of the bronchoconstriction to [βAla^8]neurokinin A(4–10) (3 nmol/kg, i.v.). At 1 µmol/kg MEN15596 inhibited the response to the selective NK_2 receptor agonist by 77±4% ($n=4$) at 5 min from antagonist administration that recovered thereafter within 60 min. At 3 µmol/kg MEN15596 induced a blockade of the response to [βAla^8]neurokinin A(4–10) within 5 min (99±1% inhibition, $n=4$) and the response was still markedly inhibited (about 80%) until 4 h.

3.6. In vivo selectivity of MEN15596 on colon contractions

The selective NK_1 receptor agonist [Sar^9]substance P sulfone (3 nmol/kg i.v.) induced colonic contractions averaging 4±1 mm Hg ($n=6$) (data not shown).

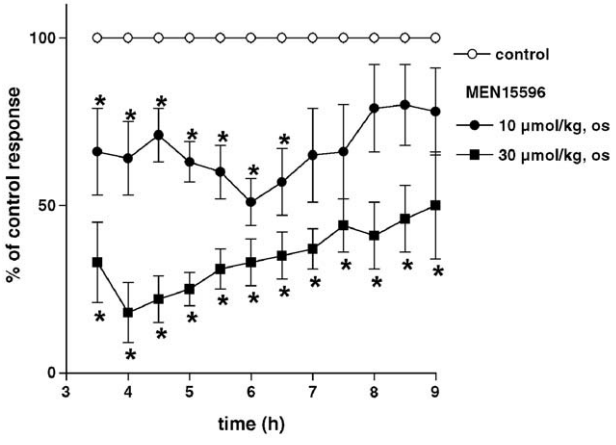

Fig. 5. Inhibitory effect of oral administration of MEN15596 (10 and 30 µmol/kg), on colon contractions induced by intravenous administration of [βAla^8]neurokinin A(4–10) (3 nmol/kg) in anaesthetized guinea-pigs. Oral administration was performed in unanaesthetized animals 45 min before anaesthesia and 3.5 h before starting of the experiment. In all experiments the animals were treated with hexamethonium bromide as a bolus (13.8 µmol/kg, i.v.) followed by continuous infusion of the same solution (300 µl/h) for the duration of the experiment. The results are expressed as % variation of the control response to [βAla^8]neurokinin A(4–10). Each value is the mean±S.E.M. of 4–10 experiments. *, $P<0.05$, significantly different from the respective value in the control group.

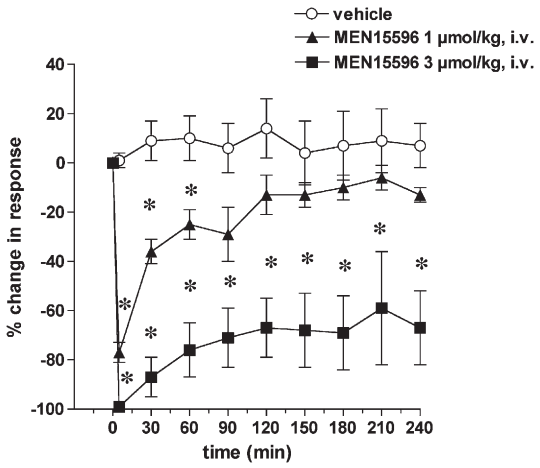

Fig. 6. Inhibitory effect of intravenous administration of MEN15596 (1–3 µmol/kg) on bronchoconstriction induced by [βAla^8]neurokinin A(4–10) (3 nmol/kg i.v.) in anaesthetized guinea-pigs. The results are expressed as % change of the control response to [βAla^8]neurokinin A(4–10) before the treatment. Each value is the mean±S.E.M. of 4–12 experiments. *, $P<0.05$, significantly different from the corresponding value in the vehicle group.

The administration of MEN15596 (3 µmol/kg) did not affect the responses produced by [Sar^9]substance P sulfone (3±1 mm Hg), whereas the response to [βAla^8]neurokinin A(4–10) (3 nmol/kg i.v.) was reduced by more than 90% (1±1 vs. 12±3 mm Hg in controls).

4. Discussion

MEN15596 is a new orally active tachykinin NK_2 receptor antagonist synthesized as a back up of the bicyclic hexapeptide antagonist, nepadutant (Catalioto et al., 1998) which is currently in clinical development. MEN15596 possesses a nonpeptide chemical structure and in comparison with the pseudopeptide nepadutant it has a lower molecular weight with only one stereogenic centre, higher potency and selectivity in blocking the human tachykinin NK_2 receptor. Notwithstanding, MEN15596 maintains some of the chemical features of nepadutant, such as aromatic groups and a basic moiety, that yielded in the past compounds with high affinity for the tachykinin NK_2 receptor (Fedi et al., 2004).

In binding experiments at the recombinant human NK_2 receptor, MEN15596 shows a very high affinity ($pK_i=10.1$), exceeding 4 and 3.7 log units the affinity found at human NK_1 and NK_3 receptors, respectively. Binding selectivity for human NK_2 receptors was confirmed when MEN15596 was tested in binding assays on more than 30 different molecular targets. Among these, MEN15596 had a measurable affinity at σ ($pK_i=6.9$), vasopressin V_{1a} ($pK_i=6.4$) and opiate receptors ($pK_i=5.6$), but a 3-fold log units margin of selectivity was maintained toward the NK_2 receptor.

The antagonist potency of MEN15596 estimated through functional experiments (Ca^{2+} mobilization) with CHO cells expressing the human NK_2 receptors was similar to that found at native human (urinary bladder, $pK_B=9.2$), guinea-pig (colon, $pK_B=9.3$), and pig (urinary bladder, $pK_B=8.8$) NK_2 receptor smooth muscle preparations. Moreover MEN15596 maintained a wide selectivity margin (>3 log units) over guinea-pig NK_1 and NK_3 receptors since the respective estimated affinity as pK_B were ≤5 and 6. Thus, both binding and functional experiments on cultured cells or isolated tissue strips indicate that MEN15596 is a potent and selective antagonist at human NK_2 receptors. Furthermore the present findings demonstrate that MEN15596 behaves as a surmountable antagonist since it does not produce a depression of the Emax according to a competitive antagonism as observed by the Schild plot analysis in guinea-pig colon and in pig urinary bladder.

Functional experiments on isolated rat and mouse urinary bladder indicate a lower affinity ($pK_B=6.3$ and 5.8, respectively) of MEN15596 in sharp contrast to what was observed with nepadutant in the same assays (pK_B 9.0 and 9.8, Catalioto et al., 1998). These species selectivity data are in agreement to what was observed with some closely related analogs of MEN15596, previously presented (Meini et al., 2004). For this a different binding epitope as compared with nepadutant, was shown by means of site-directed mutagenesis at the human NK_2 receptor, and a critical residue was suggested as responsible for this species receptor selectivity.

Given the species-related differences in the affinity of MEN15596 at NK_2 receptors, colon contractions elicited by the selective NK_2 receptor agonist in anaesthetized guinea-pigs was selected as a suitable model to assess the potency and duration of action of the compound in vivo (Giuliani et al., 1993). Indeed in this test MEN15596 produced a dose-dependent inhibition of NK_2 receptor-mediated colon contractions following both i.v. and i.d. administration. Following the i.v. administration the onset of the inhibitory effect was rapid and, especially at the lowest doses, contractions showed a time-dependent recovery indicating that the blockade of NK_2 receptors was reversible. Reversibility of MEN15596 effect was also supported by results on contractility of isolated organs, where the maximal effect induced by the agonist was never depressed in all species examined including humans, despite the rightward shifts of the concentration–response curves. In addition to the in vitro evidence, the selectivity for the NK_2 receptor has been confirmed also in *in vivo* experiments that indicate no effect on guinea pig colon contractions induced by the selective tachykinin NK_1 receptor agonist [Sar^9]substance P sulfone.

In the *in vivo* test in guinea-pigs, the onset of the inhibitory effect was also quite rapid even following the i.d. administration, since at the highest dose (10 µmol/kg) a significant inhibition was already observed at 5 min from MEN15596 administration, indicating a rapid intestinal absorption. Furthermore following the i.d. administration, MEN15596 had a long duration of effect suggesting that an oral formulation of the compound could be a candidate for the clinical development. Because of this, the activity of MEN15596 was also tested following its oral administration: the long-lasting inhibition of NK_2 receptor-mediated colonic contractions confirmed that the compound possesses a good oral activity. As reference, the absolute bioavailability of nepadutant was less than 1% following its i.d. administration and, following the oral route this

increased up to 5% when the intestinal barrier was disrupted by castor oil (Lecci et al., 2001).

Similar antagonist effects showed by MEN15596 on contractions of the guinea pig colon induced by the selective tachykinin NK_2 receptor agonist [βAla^8]neurokinin A(4–10) have been observed also in the airways *in vivo*, a system in which tachykinin NK_2 receptors play an important role (Patacchini and Maggi, 2001).

In conclusion the present results indicate that MEN15596 is a potent and selective nonpeptide tachykinin NK_2 receptor antagonist which possesses a good oral bioavailability. This feature renders MEN15596 a potential new drug for the therapy of chronic gastrointestinal disorders, such as irritable bowel syndrome, where the role of peripheral tachykinin NK_2 receptors have been demonstrated (Lecci and Maggi, 2003).

Acknowledgments

This work was supported by the Italian Ministry of University and Research, Grant No.4579.

References

Arunlakshana, A.D., Schild, H.O., 1959. Some quantitative uses of drug agonists. Br. J. Pharmacol. Chemother. 14, 45–58.

Astolfi, M., Treggiani, S., Giachetti, A., Meini, S., Maggi, C.A., Manzini, S., 1994. Characterization of the tachykinin NK_2 receptor in the human bronchus: influence of amastatin-sensitive metabolic pathways. Br. J. Pharmacol. 111, 570–574.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein–dye binding. Anal. Biochem. 75, 248–254.

Catalioto, R.-M., Criscuoli, M., Cucchi, P., Giachetti, A., Giannotti, D., Giuliani, S., Lecci, A., Lippi, A., Patacchini, R., Quartara, L., Renzetti, A.R., Tramontana, M., Arcamone, F., Maggi, C.A., 1998. MEN11420 (Nepadutant), a novel glycosylated bicyclic peptide tachykinin NK_2 receptor antagonist. Br. J. Pharmacol. 123, 81–91.

Emonds-Alt, X., Doutremepuich, J.D., Heaulme, M., Neliat, G., Santucci, V., Steinberg, R., Vilain, P., Bichon, D., Ducoux, J.P., Proietto, E., van Broeck, D., Soubrie, P., Le Fur, G., Breliere, J.C., 1993. *In vitro* and *in vivo* biological activities of SR 140,333, a novel potent nonpeptide tachykinin NK_1 receptor antagonist. Eur. J. Pharmacol. 250, 403–413.

Emonds-Alt, X., Bichon, D., Ducoux, J.P., Heaulme, M., Miloux, B., Poncelet, M., Proietto, V., Van Broeck, D., Vilain, P., Neliat, G., Soubrié, P., Le Fur, G., Brelière, J.C., 1995. SR 142801, the first potent non-peptide antagonist of the tachykinin NK3 receptor. Life Sci. 56, 27–32.

Fedi, V., Altamura, M., Balacco, G., Canfarini, F., Criscuoli, M., Giannotti, D., Giolitti, A., Giuliani, S., Guidi, A., Harmat, N.J.S., Nannicini, R., Pasqui, F., Patacchini, R., Perrotta, E., Tramontana, M., Triolo, A., Maggi, C.A., 2004. Insertion of an aspartic acid moiety into cyclic pseudopeptides: synthesis and biological characterization of potent antagonists for the human tachykinin NK-2 receptor. J. Med. Chem. 47, 6935–6947.

Giuliani, S., Barbanti, G., Turini, D., Quartara, L., Rovero, P., Giachetti, A., Maggi, C.A., 1991. NK_2 tachykinin receptors and contraction of the circular muscle of the human colon: characterization of the NK_2 receptor subtype. Eur. J. Pharmacol. 203, 365–370.

Giuliani, S., Lecci, A., Giachetti, A., Maggi, C.A., 1993. Tachykinins and reflexly evoked atropine-resistant motility in the guinea-pig colon in vivo. J. Pharmacol. Exp. Ther. 265, 1224–1231.

Grynkiewicz, G.M., Poenie, R.Y., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Jenkinson, D.H., 1991. How we describe competitive antagonists: three questions of usage. Trends Pharmacol. Sci. 12, 53–54.

Kenakin, T., 1997. Pharmacologic analysis of drug–receptor interaction, Competitive Antagonism, 3rd Edition. Lippincott–Raven Press Publishers, Philadelphia, pp. 331–373.

Kiss, S., Yoshiyama, M., Cao, Y.Q., Basbaum, A.I., de Groat, W.C., Lecci, A., Maggi, C.A., Birder, L.A., 2001. Impaired response to chemical irritation of the urinary tract in mice with disruption of the preprotachykinin gene. Neurosci. Lett. 313, 57–60.

Lecci, A., Maggi, C.A., 2003. Peripheral tachykinin receptors as potential therapeutic targets in visceral diseases. Expert Opin. Ther. Targets 7, 343–362.

Lecci, A., Carini, F., Tramontana, M., D'Aranno, V., Marinoni, E., Crea, A., Bueno, L., Fioramonti, J., Criscuoli, M., Giuliani, S., Maggi, C.A., 2001. Nepadutant pharmacokinetics and dose–effect relationships as tachykinin NK_2 receptor antagonist are altered by intestinal inflammation in rodent models. J. Pharmacol. Exp. Ther. 299, 247–254.

Lecci, A., Capriati, A., Maggi, C.A., 2004. Tachykinin NK_2 receptor antagonists for the treatment of irritable bowel syndrome. Br. J. Pharmacol. 141, 1249–1263.

Lordal, M., Navalesi, G., Theodorsson, E., Maggi, C.A., Hellstrom, P.M., 2001. A novel tachykinin NK_2 receptor antagonist prevents motility-stimulating effects of neurokinin A in small intestine. Br. J. Pharmacol. 134, 215–223.

Maghni, K., Taha, R., Afif, W., Hamid, Q., Martin, J.C., 2000. Dichotomy between neurokinin receptor actions in modulating allergic airway responses in an animal model of helper T cell type 2 cytokine-associated inflammation. Am. J. Crit. Care Med. 162, 1068–1074.

Meini, S., Patacchini, R., Giuliani, S., Lazzeri, M., Turini, D., Maggi, C.A., Lecci, A., 2000. Characterization of bradykinin B2 receptor antagonists in human and rat urinary bladder. Eur. J. Pharmacol. 388, 177–182.

Meini, S., Bellucci, F., Catalani, C., Cucchi, P., Patacchini, R., Rotondaro, L., Altamura, M., Giuliani, S., Giolitti, A., Maggi, C.A., 2004. Mutagenesis at the human tachykinin NK_2 receptor to define the binding site of a novel class of antagonists. Eur. J. Pharmacol. 488, 61–69.

Patacchini, R., Maggi, C.A., 2001. Peripheral tachykinin receptors as targets for new drugs. Eur. J. Pharmacol. 429, 13–21.

Patacchini, R., Barthò, L., Holzer, P., Maggi, C.A., 1995. Activity of SR 142801 at peripheral tachykinin receptors. Eur. J. Pharmacol. 278, 17–25.

Patacchini, R., Giuliani, S., Turini, A., Navarra, G., Maggi, C.A., 2000. Effect of nepadutant at tachykinin NK_2 receptors in human intestine and urinary bladder. Eur. J. Pharmacol. 398, 389–397.

Patacchini, R., Lecci, A., Holzer, P., Maggi, C.A., 2004. Newly discovered tachykinins raise new questions about their peripheral roles and the tachykinin nomenclature. Trends Pharmacol. Sci. 25, 1–3.

Santicioli, P., Giuliani, S., Patacchini, R., Tramontana, M., Criscuoli, M., Maggi, C.A., 1997. MEN 11420, a potent and selective tachykinin NK_2 receptor antagonist in the guinea-pig and human colon. Naunyn–Schmiedeberg's Arch. Pharmacol. 356, 678–688.

Schuiling, M., Zuidhof, A.B., Meurs, H., Zaagsma, J., 1999. Role of tachykinin NK_2 receptor activation in allergen-induced late asthmatic reaction, airway hyperreactivity and airway inflammatory cells influx in conscious, unrestrained guinea-pigs. Br. J. Pharmacol. 127, 1030–1038.

Sculptoreanu, A., de Groat, W.C., 2003. Protein kinase C is involved in neurokinin receptor modulation of N- and L-type Ca^{2+} channels in DRG neurons of the adult rat. J. Neurophysiol. 90, 21–31.

Tramontana, M., Santicioli, P., Giuliani, S., Catalioto, R.-M., Lecci, A., Carini, F., Maggi, C.A., 2002. Role of tachykinins in sephadex-induced airway hyperreactivity and inflammation in guinea-pigs. Eur. J. Pharmacol. 439, 149–158.