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MEN 11420 (Nepadutant), a novel glycosylated bicyclic peptide tachykinin NK_2 receptor antagonist

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1 The pharmacological profile was studied of MEN 11420, or cyclo{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dap-Leu]cyclo(2β-5β)}, a glycosylated derivative of the potent, selective, conformationally-constrained tachykinin NK_2 receptor antagonist MEN 10627 (cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β-5β)).

2 MEN 11420 competitively bound with high affinity to the human NK_2 receptor stably transfected in CHO cells, displacing radiolabelled [^{125}I]-neurokinin A and [^{3}H]-SR 48968 with K_i values of 2.5 ± 0.7 nM ($n=6$) and 2.6 ± 0.4 nM ($n=3$), respectively.

3 MEN 11420 showed negligible binding affinity ($pIC_{50} < 6$) at 50 different receptors (including tachykinin NK_1 and NK_3 receptors) and ion channels.

4 In the rabbit isolated pulmonary artery and rat urinary bladder MEN 11420 potently and competitively antagonized tachykinin NK_2 receptor-mediated contractions ($pK_B = 8.6 \pm 0.07$, $n=10$, and 9.0 ± 0.04, $n=12$; Schild plot slope $= -1.06$ (95% c.l. $= -1.3$; -0.8) and -1.17 (95% c.l. $= -1.3$; -1.0), respectively). MEN 11420 produced an insurmountable antagonism at NK_2 receptors in the hamster trachea and mouse urinary bladder. However, in both preparations, the effect of MEN 11420 was reverted by washout and an apparent pK_B of 10.2 ± 0.14, $n=9$, and 9.8 ± 0.15, $n=9$, was calculated in the hamster trachea and mouse urinary bladder, respectively.

5 MEN 11420 showed low affinity ($pK_B < 6$) at guinea-pig and rat tachykinin NK_1 (guinea-pig ileum and rat urinary bladder) and NK_3 (guinea-pig ileum and rat portal vein) receptors. On the whole, the affinities (potency and selectivity) showed by MEN 11420 for different tachykinin receptors, measured either in binding or in functional bioassays, were similar to those shown by the parent compound, MEN 10627.

6 The *in vivo* antagonism of the contractions produced by [βAla^8]neurokinin A(4–10) (1 nmol kg^{-1}) was observed after intravenous (dose range: 1–10 nmol kg^{-1}), intranasal (3–10 nmol kg^{-1}), intrarectal (30–100 nmol kg^{-1}) and intraduodenal (100–300 nmol kg^{-1}) administration of MEN 11420. MEN 11420 was more potent (about 10 fold) and longer lasting than its parent compound MEN 10627, possibly due to a greater metabolic stability.

7 A dose of MEN 11420 (100 nmol kg^{-1}, i.v.), that produced potent and long lasting inhibition of the contraction of the rat urinary bladder induced by challenge with the NK_2 selective receptor agonist [βAla^8]neurokinin A(4–10) (10–300 nmol kg^{-1}), was without effect on the responses produced by the NK_1 receptor selective agonist [Sar^9]substance P sulphone (1–10 nmol kg^{-1}).

8 These findings indicate that MEN 11420 is a potent and selective tachykinin NK_2 receptor antagonist. The introduction of a sugar moiety did not produce major changes in the affinity profile of this antagonist as compared to MEN 10627, but markedly improved its *in vivo* potency and duration of action. With these characteristics, MEN 11420 is a suitable candidate for studying the pathophysiological significance of tachykinin NK_2 receptors in humans.

Keywords: Tachykinins; neurokinin A; tachykinin NK_2 receptors; MEN 11420

Introduction

Neurokinin A (NKA), a member of the tachykinin peptide family, is widely distributed in the mammalian central and peripheral nervous systems: in the latter, NKA exerts its biological effects mainly by activating the tachykinin NK_2 receptor (Buck *et al*., 1984; Lee *et al*., 1986; Masu *et al*., 1987; Regoli *et al*., 1987). Tachykinin NK_2 receptor antagonists are potential candidates for the treatment of bronchial hyperreactivity, irritable bowel syndrome, cystitis and other pathological conditions putatively mediated by endogenous tachykinins: this has prompted the search for potent and selective NK_2 receptor antagonists as drug candidates (Maggi *et al*., 1993; Quartara *et al*., 1995 for reviews).

In our search for potent and selective tachykinin NK_2 receptor antagonists, we speculated that a common conformational feature should be present in NK_2 receptor agonists, such as [βAla^8]NKA(4–10) (Saviano *et al*., 1991), and certain peptide-derived antagonists, such as the cyclic hexapeptide L-659-877 (cyclo(Leu-Gln-Tpr-Phe-Gly-Met), Williams *et al*., 1988; Giolitti & Maggi, 1994). From molecular modelling studies we postulated that a β-turn around the Trp-Phe segment would be important for high affinity ligand-receptor interaction: on this basis, a conformational constraint which could lock the hypothetically active conformation in the monocyclic NK_2 receptor antagonist L-659-877 was designed (Pavone *et al*., 1995a,b). The resulting compound, MEN 10627 (cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β-5β)), bears a lactam bridge between the side chains of two amino acids and presents, therefore, a bicyclic structure which contains two consecutive β-turns (Maggi *et al*., 1994; Pavone *et al*., 1995a,b).

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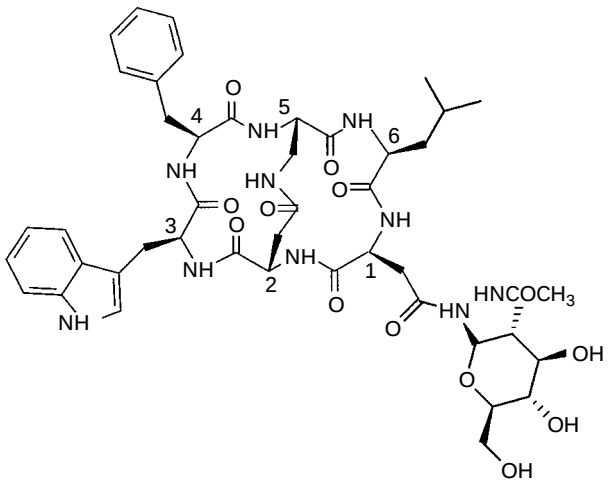

Figure 1 Chemical structure of MEN 11420.

MEN 10627 is markedly more potent than L-659-877, both *in vitro* and *in vivo* and possesses a much longer duration of action *in vivo* (Maggi *et al*., 1994). Despite these favourable characteristics, the chemical-physical properties of MEN 10627 hampered its further development as a drug candidate. Aiming at an improvement of the hydrophilicity of MEN 10627 without altering its profile of action as an NK_2 receptor antagonist, we synthesized MEN 11420, or cyclo {[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dap-Leu]cyclo(2β-5β)} (Figure 1), a bicyclic peptide bearing a (2-acetylamino-2-desoxy-β-D-glucopyranosyl)-L-asparaginyl residue in the place of the Met residue present in MEN 10627. The introduction of a glycosyl residue to increase solubility and bioavailability of peptides is an approach that has been already used in our laboratory (Pinzani *et al*., 1996), and glycopeptide analogues of substance P (SP) and of NKA receptor antagonists have been described by others (Haro *et al*., 1990; Holzemann *et al*., 1994). The sugar moiety present in MEN 11420 lends it an about 80 fold increase in water solubility as compared to MEN 10627. The aim of the present study was to describe the *in vitro* and *in vivo* pharmacology and the radioligand binding profile of MEN 11420, in comparison with the parent peptide.

Methods

Binding assays

CHO cells transfected with the human NK_2 receptor were provided by Dr J.E. Krause (Washington University, School of Medicine, St. Louis, MO). Confluent cells from 4 Petri dishes were harvested in phosphate buffered saline, pelleted by centrifugation at $200 \times g$ (4°C) and homogenized with a Polytron PT3000 (Kinematica) at 13,000 r.p.m. for 15 s, in 20 ml of 50 mM Tris-HCl buffer, pH 7.4, containing bacitracin (0.1 mg ml^{-1}), chymostatin (0.01 mg ml^{-1}), leupeptin (0.005 mg ml^{-1}) and 10 μM thiorphan (buffer A). The homogenate was centrifuged for 1 h at $25{,}000 \times g$ (4°C) and the pellet resuspended in the binding buffer, composed of buffer A supplemented with 150 mM NaCl, 5 mM $MnCl_2$ and 0.1% bovine serum albumin, at a protein concentration of about 0.35 mg ml^{-1} (Lowry *et al*., 1951). The membranes (30–50 μg protein/assay) were incubated for 30 min at 20°C with: (a) 100 pM [^{125}I]-NKA and various concentrations (0.01 nM–1 μM) of cold NKA (saturation experiments, in the absence or in the presence of 0.3, 3, 30 nM MEN 11420) or 10 different concentrations (0.01 nM–10 μM) of the competing compounds (competition experiments) or (b) 8 different concentrations of [^{3}H]-SR 48968 (0.01–10 nM) (saturation experiments, in the absence or in the presence of 0.3, 3, 30 nM MEN 11420) or 0.6 nM [^{3}H]-SR 48968 and 10 different concentrations (0.01 nM–10 μM) of the competing compounds (competition experiments), in a final volume of 0.5 ml. All the experiments were performed in duplicate or triplicate; 1 μM unlabelled NKA or 1 μM SR 48968 were used for defining nonspecific binding, when [^{125}I]-NKA or [^{3}H]-SR 48968 were used as radioligands, respectively. The reaction was terminated by the addition of 4 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4, followed by rapid filtration through Whatman GF/B filter sheets (presoaked in 0.5% bovine serum albumin for at least 3 h) with a Brandel cell harvester. The filters were washed three fold with 4 ml of the same ice-cold buffer. The trapped radioactivity was determined by use of a γ-counter (Cobra, Canberra-Packard) or a β-scintillation counter (2200 CA, Canberra-Packard).

Furthermore, the binding affinity of MEN 11420 for a range of 43 different receptors, including NK_1 and NK_3 receptors, and for Na^+, Ca^{2+} and K^+ channels (see Results) was measured, according to established methods, at Cerep (le Bois l'Eveque BP, 186600 Celle l'Evescault, France).

Organ bath studies

Male albino New Zealand rabbits (2.5–3.0 kg), male Syrian golden hamsters (100–120 g), male albino guinea-pigs (250–300 g) and male albino rats (Wistar strain, 300–350 g) were stunned and bled. Guinea-pig ileum longitudinal muscle myenteric plexus strips, rabbit pulmonary artery circular muscle strips deprived of the endothelium, rings of hamster trachea, rat urinary bladder longitudinal muscle strips and rat portal veins were prepared for recording of mechanical responses to tachykinin receptor agonists in oxygenated Krebs solution, as described in previous studies from our laboratory (Maggi *et al*., 1990; 1994; Patacchini *et al*., 1991; 1995). Furthermore, longitudinal detrusor muscle strips from the mouse urinary bladder were prepared from male albino mice (Swiss, CD-1 strain). All preparations were placed in 5 ml organ baths filled with oxygenated (96% O_2 and 4% CO_2) Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KCl 4.7 and glucose 11.

The mechanical activity of guinea-pig ileum (load 3 mN), rat urinary bladder (load 5 mN) rat portal vein (load 3 mN) and mouse urinary bladder (load 5 mN), was recorded isotonically, while that of rabbit pulmonary artery and hamster trachea (load 10 mN each) was recorded isometrically.

The activity of MEN 11420 at tachykinin NK_1 receptors was evaluated in the guinea-pig ileum (in the presence of atropine and chlorpheniramine, 1 μM each, and of indomethacin, 3 μM) and in the rat urinary bladder (in the presence of indomethacin, 10 μM), by using [Sar^9]substance P (SP) sulphone and septide (pGlu-Phe-Phe-Pro-Leu-Met-NH_2) as tachykinin NK_1 receptor selective agonists.

The activity of MEN 11420 at tachykinin NK_2 receptors of the rabbit pulmonary artery, hamster trachea and mouse urinary bladder, that are essentially monoreceptorial preparations was evaluated against NKA as the agonist. The effect of MEN 11420 at NK_2 receptors of the rat urinary bladder was studied by using [βAla^8]NKA(4–10) (Rovero *et al*., 1989) as an NK_2 receptor-selective agonist, and in the presence of the NK_1 receptor-selective antagonist SR 140333 (Emonds-Alt *et*

al., 1993) (0.1 μM) to occlude the tachykinin NK_1 receptors in this preparation (Burcher & Buck, 1986; Maggi *et al*., 1988).

The activity of MEN 11420 at tachykinin NK_3 receptors was evaluated in the guinea-pig ileum and in the rat portal vein, by using senktide (succ-[Asp^6, MePhe^8]SP(6–11)) as a tachykinin NK_3 receptor selective agonist. Atropine was not added to the medium of the guinea-pig ileum, at variance with the conditions used for studying responses mediated by the NK_1 receptor, since stimulation of prejunctional NK_3 receptors in this preparation promotes the release of acetylcholine from cholinergic nerves (Laufer *et al*., 1988) which, in turn, determines contraction via muscarinic receptors placed on smooth muscle. In these experiments SR 140333 (0.1 μM) was present in the medium, to rule out any involvement of NK_1 receptors.

In all preparations tested, cumulative concentration-response curves to the agonists were obtained, each concentration being added when the effect of the preceding one had reached a steady state. Only one concentration of antagonist was tested in each individual preparation. The preparations were challenged with the first concentration of agonist after a preincubation period of 15 min with MEN 11420. When time-dependence of the antagonist effect was investigated (i.e. with hamster trachea and mouse urinary bladder), a prolonged (120 min) preincubation period was also used.

The reversibility of MEN 11420-induced NK_2 receptor blockade was evaluated in the hamster trachea and mouse urinary bladder as follows: NKA (0.1 μM) was administered to the preparations at 30 min intervals, until reproducible contractile responses were obtained (generally after 2–3 administrations). At this time MEN 11420 was added to the bath solution, 15 min before the next challenge with the agonist. The preparations were then thoroughly washed with Krebs solution, which was renewed every 5 min. Administration of the agonist was repeated 30, 60 and 90 min after washout of the antagonist, and the responses were compared to those obtained in control time-matched preparations.

In vitro *stability tests*

Blood was collected, by cardiac puncture from ether anaesthesized rats, into heparin-treated tubes and plasma was obtained by centrifugation. The liver, the kidneys and about 40 cm of small intestine were removed, rinsed in saline and immediately homogenized, with a Polytron PT 3000 (Kinematica, Lucerne, Switzerland) homogenizer, in 9 volumes of 50 mM phosphate buffer, pH 7.5. To prepare the microsomal fraction, the liver was homogenized in 4 volumes of the buffer and centrifuged at $9000 \times g$ for 30 min; then the supernatant was centrifuged for 60 min at $105{,}000 \times g$ and the pellet was resuspended in two volumes of the buffer. The antagonists were added to the homogenates or to the microsomal suspension (with or without 1 mM NADPH) at the final concentration of 5 μM. Mixtures were incubated at 37°C and, after 6 h (1 h with microsomes), 50 μl samples were deproteinized with 250 μl of acetonitrile, vortex-mixed and centrifuged at $2{,}000 \times g$ for about 5 min. Two hundred microliters of the supernatant were evaporated to dryness under an air stream, the residue was dissolved in 100 μl of distilled water and 20 μl were subjected to high performance liquid chromatagraphic (h.p.l.c.) analysis for the determination of unchanged compounds. The h.p.l.c. system consisted of a model 465 autosampler (Kontron Instruments S.p.A., Milan, Italy) and a model LC-9A pump (Shimadzu Co., Kyoto, Japan) connected to a Nucleosil 100 C18 reversed-phase column (150×4.6 mm, particle size 5 μm, Knauer, Berlin, FRG) protected by its precolumn. Eluting peaks were detected by a fluorescence spectrophotometer (650-10S, Perkin Elmer-Hitachi, Tokyo, Japan; $\lambda_{ex} = 280$ nm and $\lambda_{em} = 350$ nm) and measured by the Class LC-10 software (Shimadzu) on a model ProLinea 3/25s Compaq personal computer. The composition of the mobile phase was: water/acetonitrile/methanol (each containing 0.1% TFA) in the ratio 60/30/10 v/v, for the analysis of MEN 11420, and 50/40/10 v/v, for the analysis of MEN 10627. The flow rate was set at 1 ml min^{-1}.

In vivo *studies*

Male albino rats Wistar strain (Charles River, Calco, Italy), weighing 340 to 400 g, were anaesthetized with urethane (1.2 g kg^{-1}, s.c.). The right jugular vein was cannulated for i.v. administration of drugs. The urinary bladder was cannulated by the transurethral route and filled with 0.5 ml of saline. The pelvic ganglia were bilaterally removed to exclude indirect components in the response to the agonists. The ureters were ligated in the middle region to keep constant the bladder volume.

In a first series of experiments the activity and selectivity of MEN 11420 (100 nmol kg^{-1}, 10 min before) was determined by checking its effect toward bladder contractions induced by i.v. administration of submaximally (50–60% of E_{max}) and maximally effective doses (see results) of the NK_1 and NK_2 receptor selective agonists [Sar^9]SP sulphone and [βAla^8]NKA(4–10), respectively. To exclude the possibility that high doses of [βAla^8]NKA(4–10) may be acting in part by stimulation of NK_1 receptors, SR 140333 (1 μmol kg^{-1}, i.v.) was administered 10 min before the first dose of the agonist.

In a second series of experiments, a submaximally effective dose of [βAla^8]NKA(4–10) (1 nmol kg^{-1}, i.v.) was used, as described previously (Maggi *et al*., 1991; 1994), to determine the NK_2 receptor blocking activity of MEN 11420 after various routes of administration as well as its duration of action. Briefly, 3 or more reproducible control responses to the agonist were elicited at 20–30 min intervals, then MEN 11420 was administered by the intravenous, intranasal, intrarectal or intraduodenal route. The challenge with the agonist was repeated at 20–30 min intervals starting 5 min after and up to 210 min from antagonist administration.

For intravenous administration, MEN 11420 was administered in saline in a volume of 0.1 ml kg^{-1}. For intranasal administration MEN 11420 was dissolved in saline: a total volume of 0.1 ml kg^{-1} was administered divided in 2 equal amounts applied to each nostril by means of a 50 μl Hamilton microsyringe, as described previously (Maggi *et al*., 1994). For intrarectal administration the rectum was emptied of the foecal pellets by an enema of 1 ml bidistilled water, then a needle for intragastric gavage (7.5 cm long) was inserted into the rectum for about 7 cm to allow the administration of the compound in a volume of 0.1 ml kg^{-1}. For intraduodenal administration, the antagonist was injected in a volume of 1 ml kg^{-1} into the proximal duodenum at about 2 cm from the pyloric sphincter.

In all experiments, vehicle-treated rats served as controls. The contractile responses were calculated as the area under the curve by using a Mini Mop apparatus (Kontron, Germany) and expressed as % variation of the control response to the agonist at various times.

Statistical analysis

All values in the text, tables or figures are means $\pm$ 95% confidence limits, or $\pm$s.e.mean. 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 Dunnett's test for multiple comparisons, when applicable. A P level <0.05 was considered statistically significant.

Competition (K_i) and saturation (K_D, B_{max}) data were analysed by EBDA/LIGAND, a nonlinear iterative fitting program (McPherson, 1985). Statistical analysis was made by using the computer programme InStat for Macintosh (GraphPad Software).

For organ bath experiments, the competitive nature of antagonism was checked by the Schild analysis. Antagonists providing plots with linear regression lines and slopes not significantly different from unity were considered competitive. The affinity of competitive antagonists was expressed in terms of pK_B (negative logarithm of the antagonist dissociation constant), estimated according to the equation: $pK_B = \log_{10}$ [dose-ratio -1] $-\log_{10}$ [antagonist concentration], assuming a slope value of -1 (Kenakin, 1993; Jenkinson, 1991).

In the hamster trachea and mouse urinary bladder MEN 11420 caused nonparallel rightward shifts of the concentration-response curves to NKA, and decreased the E_{max}. Thus, the affinity of MEN 11420 for NK_2 receptors in these preparations was estimated by the double-reciprocal plot method described by Kenakin (1993) for noncompetitive and/or pseudoirreversible antagonists. In practice, a double-reciprocal plot of equieffective concentrations of agonist (A) in the absence (1/A) and in the presence (1/A′) of the antagonist (B) was constructed and K_B was derived from the equation: $K_B = [B]/(\text{slope} - 1)$

Drugs and solutions

[^{125}I]-NKA was purchased from Amersham (Amersham, U.K.), [^{3}H]-SR 48968 ((**S**)-*N*-methyl-*N*-*N*-[4-acetylamino-4-phenylpiperidino) - 2 - (3,4 - dichlorophenyl) -butyl]-benzamide) was purchased from DuPont-NEN (Boston, U.S.A.). MEN 11420 (cyclo{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dap-Leu]cyclo(2β-5β)}), MEN 10627 (cyclo[(Met-Asp-Trp-Phe-Dpr-Leu)cyclo(2β-5β)]), NKA and [βAla^8]NKA(4–10) were synthesized at Menarini laboratories, Florence, Italy, by conventional solid-phase methods. The nonpeptide antagonist SR 140333, [(**S**)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy-phenylacetyl) piperidin - 3 - yl] ethyl} - 4 - phenyl - 1 - azoniabicyclo[2,2,2]octane chloride], was kindly provided by Drs X. Emonds-Alt and G. Le Fur (Sanofi Recherche, Montpellier, France). Other drugs used were: indomethacin, chlorpheniramine maleate (Sigma, St. Louis, MO, U.S.A.); atropine sulphate (Serva, Heidelberg, F.R.G.); senktide, septide and [Sar^9]SP sulphone (Peninsula Laboratories, St. Helens, U.K.).

For *in vivo* experiments stock solutions of MEN 11420 (1 mM) were prepared in saline. [βAla^8]NKA(4–10) was dissolved in DMSO (3 mM), then diluted in DMSO for the dose-response curve (volume of injection 0.1 ml kg^{-1}) or in saline for the repeated single-dose protocol (volume of administration 1 ml kg^{-1}).

Results

Binding at the human NK_2 receptor in transfected CHO cells

Competition experiments MEN 11420 showed high affinity for the human NK_2 receptor expressed in CHO cells, with the same K_i value whatever the radioligand. Its affinity was comparable with that of MEN 10627, when [^{3}H]-SR 48968 was used, and only about four fold lower than that of the parent peptide with [^{125}I]-NKA as the radioligand (Table 1).

Saturation experiments MEN 11420 concentration-dependently increased the K_D of either [^{125}I]-NKA or [^{3}H]-SR 48968, without significantly affecting the B_{max} (Tables 2 and 3, Figure 2), a behaviour appropriate for a competitive ligand.

Receptor selectivity and ion channel binding affinity The affinity of MEN 11420 was measured at a variety of tachykinin and non-tachykinin receptors and of ion channels. MEN 11420 showed negligible binding affinity ($pIC_{50} < 6.0$) at NK_1 and NK_3, B_1 and B_2, bombesin, calcitonin gene-related peptide (CGRP), CCK_A and CCK_B, α_1 and α_2, β_1 and β_2, D_1 and D_2, ET_A and ET_B, $GABA_A$ and $GABA_B$, galanin, AMPA, kainate, NMDA, glycine, H_1, H_2, H_3, muscarinic, NPY,

Table 1 Affinities of MEN 11420 and MEN 10627 for the human NK_2 receptor expressed in CHO cell membranes labelled with agonist and antagonist radioligands

	K_i (nM)	
	[^{3}H]-SR 48968	[^{125}I]-NKA
Antagonists		
MEN 11420	2.6±0.4	2.5±0.7*
MEN 10627	1.8±0.3	0.6±0.4

Mean K_i (equilibrium inhibition constant) values±s.e.mean are presented. Data were obtained from 3–6 separate competition experiments, each one performed in duplicate. *$P<0.05$, versus MEN 10627.

Table 2 Effects of MEN 11420 on the binding of [^{125}I]-NKA to CHO/hNK_2 cell membranes

Concentration	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n_H
0	2.4±0.7	323±65	1.01±0.2
0.3 nM	2.6±0.54	259±19	1.02±0.03
3 nM	4.5±0.06	255±38	1.0±0.01
30 nM	16.3±4.4*	199±24	0.92±0.06

K_D (equilibrium dissociation constant), B_{max} (maximum binding site density) and n_H (Hill slope coefficient) are presented as means±s.e.mean of 3 separate saturation experiments, performed in triplicate. *$P<0.05$ compared to the data obtained in the absence of MEN 11420.

Table 3 Effects of MEN 11420 on the binding of [^{3}H]-SR 48968 to CHO/hNK_2 cell membranes

Concentration	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n_H
0	0.22±0.01	2359±391	0.97±0.05
0.3 nM	0.26±0.03	2368±324	1.07±0.08
3 nM	0.48±0.05	2460±146	0.99±0.02
30 nM	1.22±0.13*	2088±347	1.06±0.01

K_D (equilibrium dissociation constant), B_{max} (maximum binding site density) and n_H (Hill slope coefficient) are presented as means±s.e.mean of 3 separate saturation experiments, performed in triplicate. *$P<0.05$ compared to the data obtained in the absence of MEN 11420.

neurotensin, nicotinic, opiate, PCP, P_{2X}, P_{2Y}, 5-HT_{1A}, 5-HT_{1B}, $5HT_{1D}$, 5-HT_{2A}, 5-HT_3, 5-HT_4, σ, somatostatin and VIP receptors. Nor had MEN 11420 (1 μM) measurable affinity for Na^+ (site 1 and 2), Ca^{2+} (L and N types) or K^+ (ATP-, voltage- and Ca^{2+}-dependent) channels (data not shown).

Organ bath experiments

Effect of MEN 11420 at tachykinin NK_2 receptors in isolated organs from various species The mouse isolated urinary bladder is a preparation in which tachykinins produce a contraction by exclusive stimulation of NK_2 receptors. Neither the NK_1 receptor selective agonists, [Sar^9]SP sulphone and septide (1 μM each), nor the NK_3 receptor selective agonist senktide (1 μM) produced responses in this mouse tissue ($n=4$ for each agonist), while NKA induced concentration-dependent contraction ($EC_{50}=55$ nM (95% c.l. = 47–65), $n=18$) (Figure 3). After equilibration (1 h), the concentration-response curve to NKA was reproducible over an experimental period of at least 4 h if elicited at 1 h interval with intervening washout.

Up to 10 μM, MEN 11420 did not produce agonist effects in any of the bioassays tested (rabbit pulmonary artery, hamster trachea, rat urinary bladder, mouse urinary bladder, guinea-pig ileum and rat portal vein). MEN 11420 produced a concentration-dependent rightward shift of the log concentration-response curve to the agonist in the rabbit pulmonary artery and in the rat urinary bladder without inducing depression of E_{max}: in both preparations, Schild plot was compatible with competitive antagonism at tachykinin NK_2 receptors (Figure 4).

In the hamster trachea and mouse urinary bladder MEN 11420 produced a concentration-dependent non parallel rightward shift of the curve to the agonist while depressing E_{max} (Figure 3). To investigate whether the observed insurmountable antagonism could result from an irreversible interaction with hamster or mouse NK_2 receptors, we evaluated the reversibility and the time-dependence of the receptor blockade induced by MEN 11420 in these preparations. As shown in Figure 5, the inhibition exerted by MEN 11420 (10 nM, 15 min incubation) on the response produced by NKA (100 nM) in the hamster trachea was fully and quickly reversed by washout. Neither the E_{max} of NKA nor the extent of the rightward shift of the curve to NKA were significantly modified by prolonging the incubation of MEN 11420 (3 nM) from 15 to 120 min, respectively (Figure 5).

In the mouse urinary bladder the inhibition exerted by MEN 11420 (10 nM, 15 min incubation) on the response produced by NKA (100 nM) was slowly but fully reversed by washout (Figure 5). Prolonging the contact time of MEN 11420 from 15 to 120 min slightly increased the antagonist potency of MEN 11420 (Figure 5).

Since the insurmountable antagonism exerted by MEN 11420 in the hamster trachea and mouse urinary bladder is not a consequence of an irreversible interaction with hamster or mouse NK_2 receptors, the apparent pK_B values for the above receptors were estimated by the double-reciprocal plot method (see Methods). The data shown in Table 4 compare the apparent pK_B values of MEN 11420 and MEN 10627 at NK_2 receptors expressed in the four preparations under study: only minor differences ($\leqslant 0.5$ log units) in pK_B values were observed

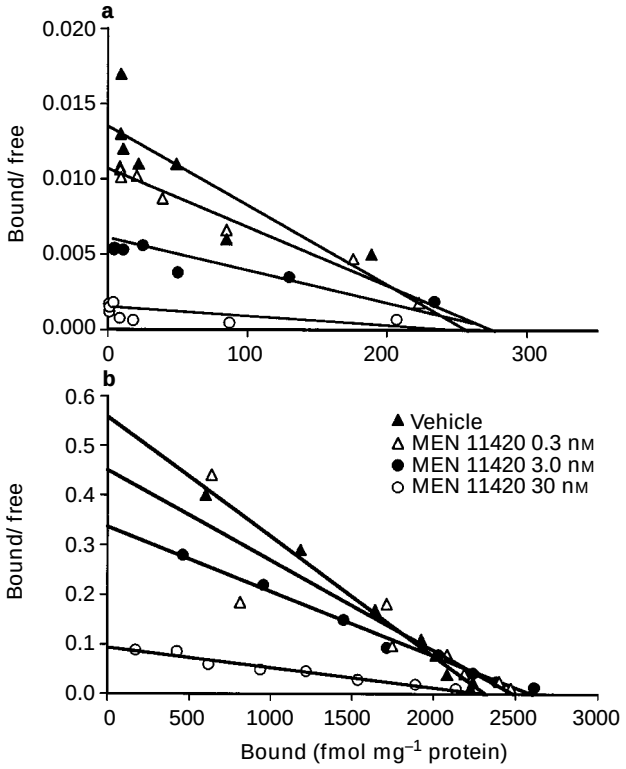

Figure 2 Effects of MEN 11420 on the binding of [^{125}I]-NKA (a) and [^{3}H]-SR 48968 (b) to membranes of CHO cells transfected with human NK_2 receptor (Scatchard plots). Each curve shows a representative saturation experiment ($n=3$) performed in triplicate, in the presence of vehicle or of MEN 11420.

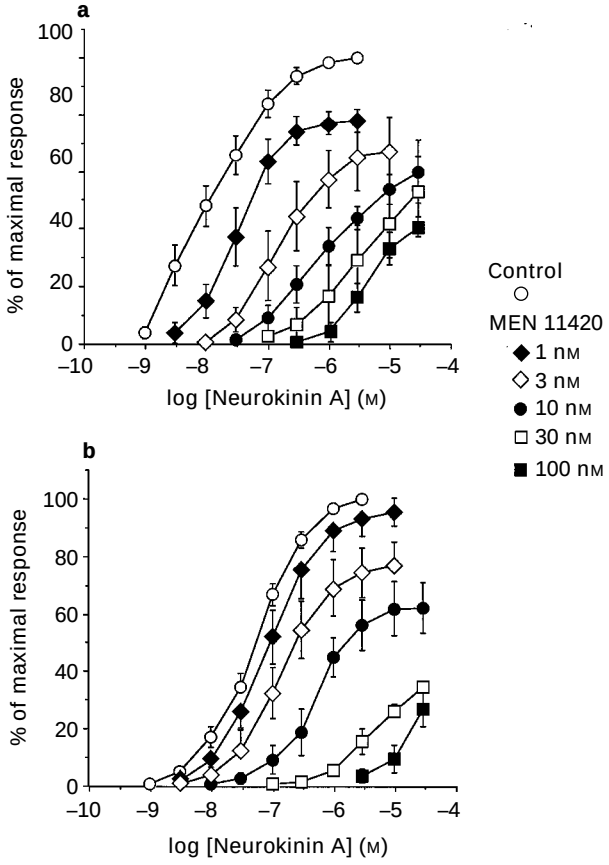

Figure 3 Antagonism by MEN 11420 at hamster and mouse tachykinin NK_2 receptors. Log concentration-response curves to NKA are shown in (a) the hamster trachea and (b) the mouse isolated urinary bladder in the absence and presence of MEN 11420. Each point is the mean of 3–4 experiments; vertical lines show s.e.mean.

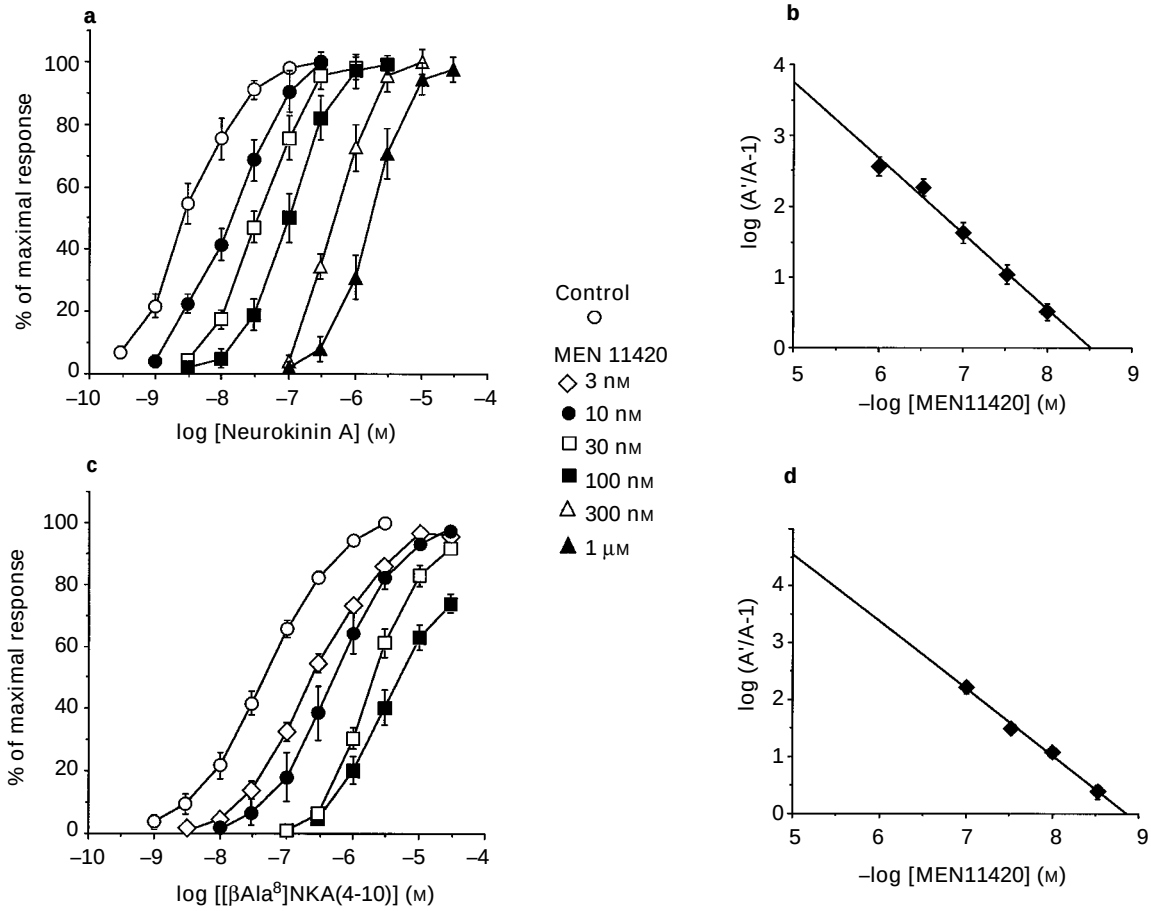

Figure 4 Antagonism by MEN 11420 at rabbit and rat tachykinin NK_2 receptors. (a) Log concentration-response curves for NKA in the rabbit isolated pulmonary artery in the absence and presence of MEN 11420. Each point is the mean of 3–4 experiments. (b) Corresponding Schild plot of agonist dose-ratios vs MEN 11420 concentrations in the rabbit isolated pulmonary artery (slope = −1.06 (95% c.l. = −1.3; −0.8)). Each point is the mean of 3–4 experiments. (c) Concentration-response curves for [βAla^8]NKA (4–10) in the rat urinary bladder in the absence and presence of MEN 11420. All the experiments were performed in the presence of SR 140333 0.1 μM. Each point is the mean of 3–4 experiments. (d) Corresponding Schild plot of agonist dose-ratios vs MEN 11420 concentrations in the rat isolated urinary bladder (slope = −1.17; 95% c.l. = −1.3; −1.0). Each point is the mean of 3–4 experiments. In (a–d), vertical lines show s.e.mean.

between MEN 11420 and MEN 10627 in the various species examined.

Effect of MEN 11420 at tachykinin NK_1 and NK_3 receptors
Data shown in Figure 6 and Table 5, indicate that MEN 11420 produces a weak competitive antagonism ($pK_B < 6$) toward NK_1 receptor-mediated contractions in the guinea-pig ileum when either septide or [Sar^9]SP sulphone were used as agonists. When assayed at rat NK_1 receptors of the rat urinary bladder, MEN 11420 (10 μM) showed no appreciable antagonism toward septide- or [Sar^9]SP sulphone-induced contractions (Table 5).

In the guinea-pig ileum preparation set up for recording NK_3 receptor-mediated responses to tachykinins (senktide as agonist, SR 140333 present in the medium) MEN 11420 produced a weak ($pK_B < 6$) competitive antagonism of the response to senktide (Table 5; Figure 6). Up to 10 μM MEN 11420 failed to antagonize senktide-mediated contractions in the rat portal vein (Table 5).

In vitro *stability tests*

MEN 11420 was remarkably stable to enzymatic degradation in all the media tested, since less than 10% of the added compound was lost at the end of both a 6 h-incubation with enzymes in plasma and rat tissue homogenates, and a 1 h-incubation with cytochrome P450 oxygenases in liver microsomes (Table 6). On the other hand, MEN 10627, which resisted the hydrolytic activities present in rat plasma and kidneys homogenate, was slowly degraded by liver and small intestine homogenates and by the hepatic NADPH-dependent microsomal enzymes, with a loss of up to 70% of the original amount at the end of the incubation periods (Table 6).

In vivo *experiments*

In an initial series of experiments we used a submaximal and a maximal dose of the NK_1 and NK_2 receptor selective agonists, [Sar^9]SP sulphone and [βAla^8]NKA(4–10), respectively, to produce contraction of the rat urinary bladder *in vivo* and to verify the effectiveness and selectivity of MEN 11420 (100 nmol kg^{-1}, i.v., 10 min before) as an NK_2 receptor antagonist. A maximally effective dose of [Sar^9]SP sulphone (10 nmol kg^{-1}, i.v.) and [βAla^8]NKA(4–10) (300 nmol kg^{-1}, i.v.) increased bladder pressure by 58 ± 3 and 46 ± 7 mmHg, respectively ($n = 5$). Ten minutes after the administration of MEN 11420 (100 nmol kg^{-1}, i.v.) the response to [Sar^9]SP

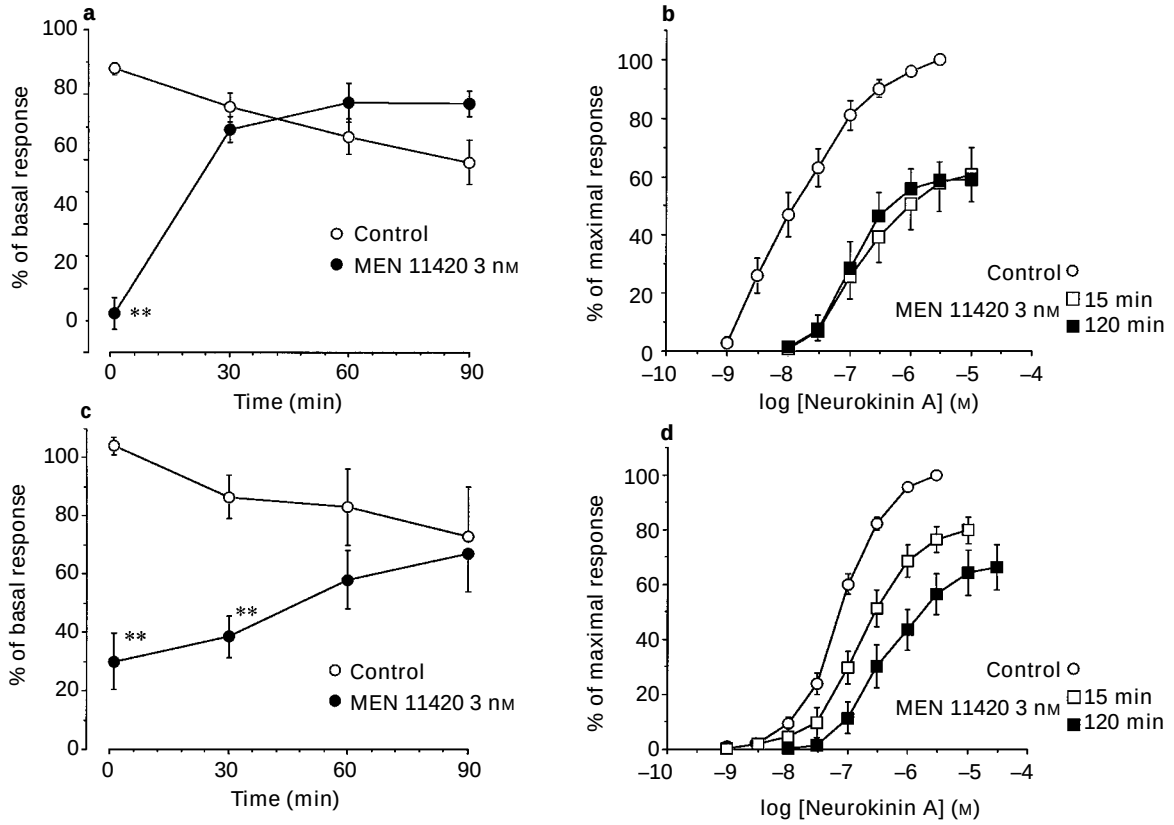

Figure 5 Reversibility and time-dependence of tachykinin NK_2 receptor blockade induced by MEN 11420 in the hamster isolated trachea and mouse isolated urinary bladder. (a and c) The contractile response to single doses of NKA (100 nM) is shown in the absence and presence (time = 0) of MEN 11420 (10 nM; 15 min incubation), and after 30, 60, and 90 min from washout of the antagonist in the hamster trachea and mouse urinary bladder, respectively. Each point is the mean of 4–6 determinations; vertical lines show s.e.mean. **Significantly different from the corresponding control response obtained after incubation with vehicle; $P<0.01$. (b and d) Concentration-response curves to NKA are shown in the absence of MEN 11420 and after 15 or 120 min incubation with MEN 11420 (3 nM) in the hamster trachea and mouse urinary bladder, respectively. Each point is the mean of 4–5 experiments; vertical lines show s.e.mean.

Table 4 Antagonist activity of MEN 11420 and MEN 10627 at tachykinin NK_2 receptors in isolated organ bioassays from different species

Antagonist	*Rabbit pulmonary artery*	*Hamster trachea*	*Rat urinary bladder*	*Mouse urinary bladder*
MEN 11420	8.6 ± 0.07	10.2 ± 0.14[a]	9.0 ± 0.04	9.8 ± 0.15[a]
MEN 10627	8.1 ± 0.1[b]	10.1 ± 0.10[b]	8.8 ± 0.08[b]	10.3 ± 0.14[a]

Each value is presented as the means ± s.e.mean ($n=9-12$) of pK_B, (negative logarithm of the antagonist dissociation constant) calculated from the Schild plot in the evidence of competitive antagonism or [a]by the double reciprocal plot method (see Methods) for noncompetitive or pseudoirreversible antagonism. [b]The data are from Maggi *et al.* (1994).

sulphone (49 ± 5 mmHg, $n=5$) was not significantly different from control while that to [βAla^8]NKA(4–10) was markedly reduced (5 ± 1 mmHg, $n=5$, $P<0.05$). Similar results were obtained when a dose of the agonists which produced about 50–65% of the maximal response was used. [Sar^9]SP sulphone (1 nmol kg^{-1}, i.v.) and [βAla^8]NKA(4–10) (10 nmol kg^{-1}, i.v.) increased bladder pressure by 30 ± 6 and 30 ± 3 mmHg ($n=5$), respectively, before administration of MEN 11420, and by 30 ± 4 (NS) and 1 ± 0.1 mmHg ($P<0.05$) ($n=5$), respectively, after administration of MEN 11420 (100 nmol kg^{-1}, i.v. 10 min before).

In subsequent experiments, a dose of [βAla^8]NKA(4–10) (1 nmol kg^{-1}) which produced a response of 12.9 ± 0.9 mmHg ($n=84$), approximately 30% of the maximal response at 300 nmol kg^{-1}, was selected: this response did not show significant variations up to 210 min from the vehicle (saline) administration. Intravenous administration of MEN 11420 (0.3–10 nmol kg^{-1}) produced a dose-dependent inhibition of the responses of the rat urinary bladder to [βAla^8]NKA(4–10) (Figure 7a). MEN 11420 showed a long duration of action: no sign of recovery was observed up to 210 min from administration of MEN 11420 at doses of 1–10 nmol kg^{-1}. When these data were compared with those obtained with MEN 10627 in the same model (Maggi *et al.*, 1994), MEN 11420 appeared to be about 10 fold more potent than its parent compound and to possess a longer duration of action: as an example, 1 nmol kg^{-1} MEN 11420 produced >60% inhibition of the response to the agonist, and no fading was evident up to 210 min. In contrast, a 10 nmol kg^{-1} MEN 10627 was required to produce a

comparable inhibitory effect which lasted only up to 120 min from i.v. administration of the antagonist (Maggi *et al.*, 1994).

As shown in Figure 7, MEN 11420 was active at inhibiting urinary bladder contractions induced by i.v. [βAla^8]NKA(4–10) also after intranasal, intrarectal and intraduodenal administration. A significant inhibitory effect towards the response of the agonist was produced by MEN 11420 at doses of 3–10, 30–100 and 100–300 nmol kg^{-1} after intranasal, intrarectal and intraduodenal administration, respectively. For

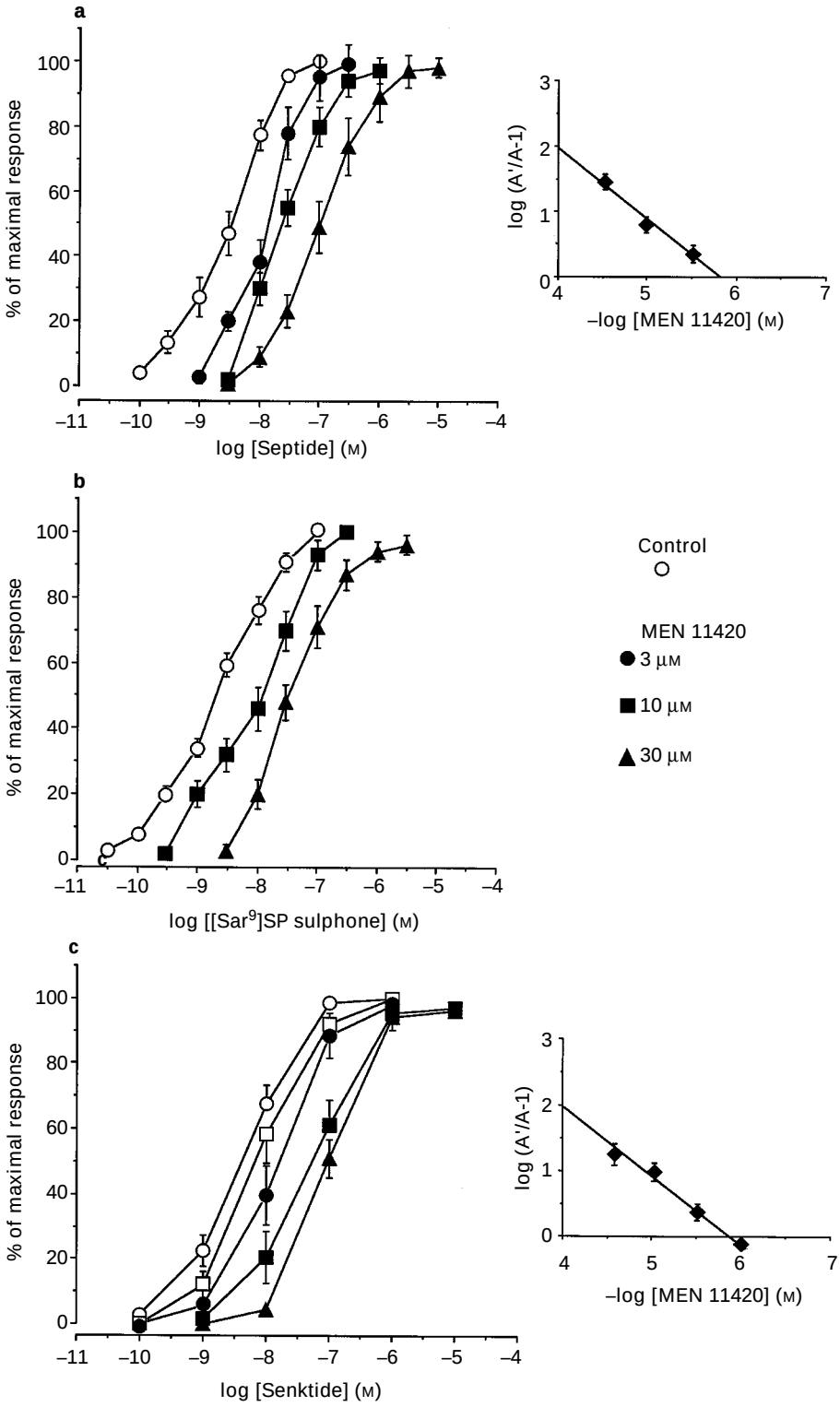

Figure 6 Antagonism by MEN 11420 at guinea-pig tachykinin NK_1 and NK_3 receptors. (a) Concentration-response curves for septide in the guinea-pig isolated ileum in the absence and presence of MEN 11420. In the inset there is the corresponding Schild plot of agonist dose-ratios vs MEN 11420 concentrations (slope = −1.05 (95% confidence limits = −1.3; −0.8)). (b) Concentration-response curves for [Sar^9]substance P sulphone in the absence and in the presence of MEN 11420. (c) Concentration-response curves for senktide in the guinea-pig ileum, in the absence and presence of MEN 11420. All the experiments were performed in the presence of SR 140333 0.1 μM (15 min before). In the inset there is the corresponding Schild plot of agonist dose-ratios vs MEN 11420 concentrations (slope = −1.05 (95% c.l. = −1.5; −0.6)). In (a–c) each point is the mean of 3–4 experiments; vertical lines show s.e.mean.

all these three routes of administration, the onset of the blockade was slow (60–120 min to peak inhibition) and the activity was sustained (little or no sign of recovery of the response to the agonist up to 210 min from antagonist administration).

Again, a comparison with MEN 10627 (Maggi *et al*., 1994) indicates a marked improvement of *in vivo* activity of MEN 11420 after intranasal and intraduodenal administration. For example, after intranasal administration, doses of 10 and 100 nmol kg^{-1} of MEN 11420 and MEN 10627 were, respectively, required to produce similar inhibition (about 80%) of the response to the agonist. Moreover, whilst the effect of MEN 11420 showed no fading up to 210 min from its intranasal administration (Figure 7b), the effect of MEN 10627 showed a significant fading at >120 min from its administration (Maggi *et al*., 1994).

Table 5 Antagonist activity of MEN 11420 and MEN 10627 at tachykinin NK_1 and NK_3 receptors present in guinea-pig and rat isolated bioassays

	NK_1		*NK_3*	
Antagonist	*Guinea-pig ileum*	*Rat urinary bladder*	*Guinea-pig ileum*	*Rat portal vein*
MEN 11420	5.9 ± 0.04[a]/5.7 ± 0.04[b]	<5[a,b]	5.9 ± 0.08	<5
MEN 10627	6.1 ± 0.12[c,d]	<6[d]	NV[d,e]	<5[d]

Each value is the mean ± s.e.mean ($n=9-12$) of pK_B (negative logarithm of the antagonist dissociation constant). NK_1 receptor-selective agonists used in the guinea-pig ileum were [a]septide, [b][Sar^9]SP sulphone and [c]SP methylester. [d]Data are from Maggi *et al.* (1994). [e]NV: insurmountable antagonism, pK_B not valid.

Table 6 *In vitro* metabolic stability of MEN 11420 and MEN 10627 in different media

		Rat tissue homogenates			*Rat liver microsomes*	
Antagonist	*Rat plasma*	*Liver*	*Kidneys*	*Small intestine*	*+NADPH*	−NADPH
MEN 11420	>90%	>90%	>90%	>90%	>90%	>90%
MEN 10627	>90%	38%	>90%	31%	35%	>90%

Data are presented as % of original amounts remaining after 6 h incubation in plasma and homogenates or after 1 h incubation in microsomal suspension. Tests were performed in duplicate.

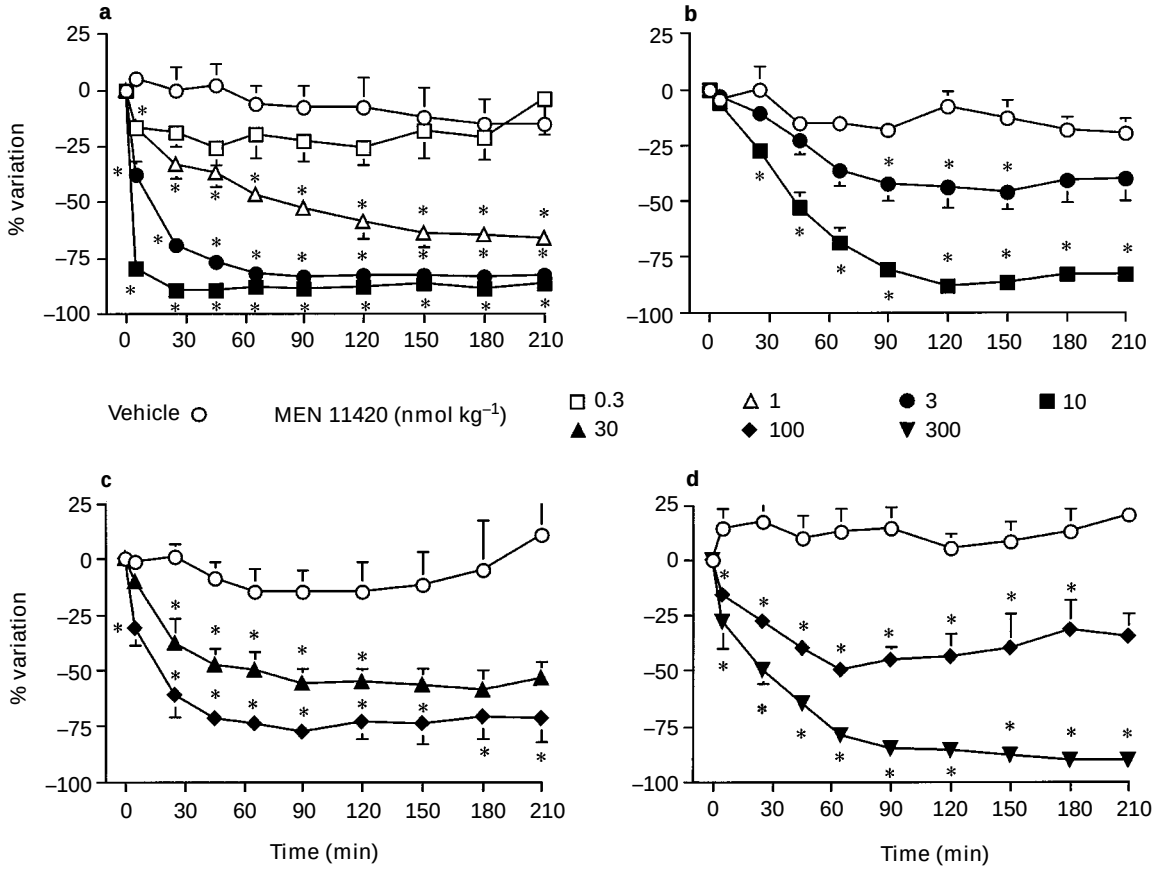

Figure 7 Inhibitory effect of MEN 11420, administered through different routes, on the urinary bladder contractions induced by [βAla^8]NKA(4–10) (1 nmol kg^{-1}, i.v.) in urethane anaesthetized rats. After 2 or more reproducible control responses to the agonist had been established at 20 min interval, the antagonists or the vehicle were administered either intravenously (a), intranasally (b), intrarectally (c) or intraduodenally (d). Responses are expressed as % variation of the control response to the agonist. *Significantly different from the respective vehicle group ($P<0.05$, Dunnett's test for multiple comparison).

The improved *in vivo* activity of MEN 11420 was even more evident after intraduodenal administration: a long lasting 50% inhibitory effect of MEN 11420 was observed at the dose of 100 nmol kg^{-1} (Figure 7d). In the same assay, MEN 10627 was shown to need a dose of 3 μmol kg^{-1} to produce an inhibition of about 40%, the activity being no longer significant beyond 180 min from antagonist administration (Maggi *et al*., 1994).

Discussion

The present findings demonstrate that the modifications on the side chain of the Met residue of MEN 10627 (Maggi *et al*., 1994), with the introduction of a glycosylated moiety, do not markedly affect the potency as a tachykinin NK_2 receptor antagonist *in vitro*. Both MEN 10627 and MEN 11420 are potent NK_2 receptor antagonists, yet the latter compound showed more favourable chemical-physical characteristics, as demonstrated by the increase (about 80 fold) in water solubility. Also the good selectivity of the compound was preserved following the introduction of the glycosylated moiety: the affinity of MEN 11420 for rat NK_2 receptors ($pK_B = 9.0$) was about 4 orders of magnitude higher than for rat NK_1 or NK_3 receptors, a profile similar to that of MEN 10627. MEN 11420 showed no detectable binding affinity for a large number of non-tachykinin receptors and ion channels. This characteristic distinctly separates this class of compounds from non-peptide tachykinin receptor antagonists, such as SR 48968, which possesses sizeable affinity for μ opioid receptors (Martin *et al*., 1992; Boyle *et al*., 1993) and local anaesthetic-like activity (Wang *et al*., 1995). It appears likely that the highly constrained backbone of MEN 10627 and MEN 11420 (Pavone *et al*., 1995a,b) which is optimized for interacting with NK_2 receptors, does not easily enable it to fit into other binding sites. A similar explanation may, at least in part, account for the resistance to metabolic degradation showed by MEN 10627 and, especially, MEN 11420. The absence of measurable *in vitro* metabolism for the latter compound may be also related to the substitution of the oxidable methionine residue present in MEN 10627.

MEN 11420 exerted a competitive type of interaction at human NK_2 receptors transfected in CHO cells when either a peptide agonist (NKA) or a nonpeptide antagonist (SR 48968) was used as a radioligand. A partial overlapping between the binding sites of the parent compound MEN 10627 and SR 48968 is suggested by the observation that co-incubation of these antagonists prevents the depressant effect of SR 48968 on the attainment of E_{max} to the agonist in functional assays (Patacchini *et al*., 1994). Clearly a mutational mapping analysis of the NK_2 receptor is required to establish to what extent a peptide derived antagonist, such as MEN 11420, could share the binding site(s) of natural agonist and nonpeptide antagonist on the human NK_2 receptor.

Similar to many other tachykinin receptor antagonists of peptide and non-peptide nature (Maggi *et al*., 1990; 1993), MEN 11420 also displayed remarkable differences in affinity for NK_2 receptors expressed in different species. The molecular basis for these differences has not yet been ascertained, although they probably originate from species-dependent variations at nonconserved positions in the primary sequence of the NK_2 receptor protein. Notably, the pattern of species-dependent variable affinities of MEN 11420 does not differ from that of MEN 10627, further suggesting that the sugar moiety introduced in MEN 11420 does not directly interact with the receptor.

Interestingly, MEN 11420 produced a clearcut depression of the E_{max} to the agonist in the hamster trachea and mouse urinary bladder assays: this behaviour occurs in preparations in which an exceedingly high affinity of the ligand for NK_2 receptors has been measured (apparent $pK_B = 10.2$ and 9.8) and its origin is uncertain. It has to be noted that a competitive behaviour has been observed for MEN 10627 in the hamster trachea assay (Maggi *et al*., 1994), which is different from the noncompetitive behaviour observed for MEN 11420: it is possible that the different structure of the two antagonists differentially interacts with the microenvironment of the cell membrane in the surroundings of the NK_2 receptor. While further studies are needed to address this issue by, for example, investigating the pattern of glycosylation of the NK_2 receptor in different species, we can exclude the possibility that an irreversible interaction with the receptor had occurred, since depression of the response to the agonist by MEN 11420 was fully reversible upon washout in both hamster trachea and mouse urinary bladder.

The most striking pharmacological advantages of MEN 11420 over MEN 10627 were evident *in vivo*: the two compounds have similar affinities for NK_2 receptors in the rat urinary bladder smooth muscle (pK_B 9.0 and 8.8, respectively) yet, after intravenous administration, MEN 11420 was about 10 fold more potent than MEN 10627 in inhibiting the contraction produced by the NK_2 receptor selective agonist and its blocking activity had a longer duration of action than that of MEN 10627. The improved pattern of *in vivo* antagonist activity relates to a more persistent presence of MEN 11420 in plasma, as compared to MEN 10627 (unpublished data), which, in turn, is probably related to the more favourable chemical physical characteristics and to the lower liability to metabolic elimination. Moreover, despite the increase in molecular size (m.w. 947 and 761 for MEN 11420 and MEN 10627, respectively) the improvement of *in vivo* activity was also evident after intranasal and intraduodenal administration of the compounds, indicating that the presence of the sugar moiety in MEN 11420 increases bioavailability. On comparison of the doses required to produce inhibition of the urinary bladder contraction in response to an NK_2 receptor agonist after intraduodenal administration in anaesthetized rats, MEN 11420 appeared to be about 30 fold more potent than MEN 10627 and about 10 fold more potent than the nonpeptide antagonist SR 48968 (see Maggi *et al*., 1994). In those studies, MEN 10627 had been administered in 5% carboxymethylcellulose/4% Tween 80, because of its very poor water solubility, but the difference in vehicle cannot account for the greater bioavailability of MEN 11420, since its effects after intraduodenal administration are not changed if it is dissolved exactly as MEN 10627 (data not shown).

Despite its good activity after intraduodenal administration, higher doses of MEN 11420 are needed to produce blockade of the NK_2 receptors than after i.v. administration. This difference was much less pronounced if the compound was administered by the intranasal route, indicating a very good systemic bioavailability following this route of administration. The nasal mucosa has proven to be the most promising, among absorptive mucosae investigated so far, for the delivery of peptide and protein-based drugs that are not readily available by the oral route (Sayani & Chien, 1996). However, the presence of an active enzymatic barrier may sometimes reduce the practicability of this route (Sarkar, 1992). The high bioavailability of nasally instilled MEN 11420, probably related to its low liability to enzymatic degradation, suggests that the intranasal route might be conveniently exploited for a noninvasive parenteral formulation of the compound for its clinical investigation.

In conclusion, the present findings indicate that MEN 11420 is a potent and selective tachykinin NK_2 receptor antagonist, with a markedly improved profile of action compared to its lipophilic parent compound MEN 10627. Owing to the combination of its *in vitro* and *in vivo* pharmacological properties MEN 11420 appears to be a very useful tool for probing the pathophysiological role of peripheral tachykinin NK_2 receptors in man.

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