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Mutagenesis at the human tachykinin NK_2 receptor to define the binding site of a novel class of antagonists

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Abstract

The pharmacological profile of novel antagonists endowed with high affinity for the human tachykinin NK_2 receptor is presented. MEN13918 (Nγ{Nα[Nα(benzo[*b*]thiophen-2-yl)carbonyl]-1-aminocyclohexan-1-carboxy]-D-phenylalanyl}-3-*cis*-aminocyclohexan-1-carboxylic-acid-*N*-(1*S*,2*R*)-2-aminocyclohexyl)amide trifluoroacetate salt) and MEN14268 (Nα[Nα(benzo[*b*]thiophen-2-yl)carbonyl)-1-aminocyclopentane-1-carboxyl]-D-phenylalanine-*N*-[3(morpholin-4-yl)propyl]amide trifluoroacetate salt) were more potent in blocking neurokinin A (NKA, His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) induced contraction in human, which induced greater contraction in human (pK_B 9.1 and 8.3) than rat (pK_B 6.8 and <6) urinary bladder smooth muscle preparation in vitro. In agreement with functional data, in membrane preparations of CHO cells stably expressing the human NK_2 receptors, both MEN13918 and MEN14268 potently inhibited the binding of agonist ([^{125}I]NKA, K_i 0.2 and 2.8 nM) and antagonist ([^{3}H]nepadutant, K_i 0.1 and 2.2 nM, [^{3}H]SR48968 K_i 0.4 and 6.9 nM) radioligands. Using site-directed mutagenesis and radioligands binding we identified six residues in the transmembrane (TM) helices that are critical determinants for the studied antagonists affinity. To visualize these experimental findings, we constructed a homology model based on the X-ray crystal structure of bovine rhodopsin and suggested a possible binding mode of these newly discovered antagonist ligands to the human tackykinin NK_2 receptor. Both MEN13918 and MEN14268 bind amongst TM4 (Cys167Gly), TM5 (Tyr206Ala), TM6 (Tyr266Ala, Phe270Ala), and TM7 (Tyr289Phe, Tyr289Thr). MEN13918 and MEN14268 diverging binding profile at Y289 mutations in TM7 (Tyr289Phe, Tyr289Thr) suggests a relation of their different chemical moieties with this residue. Moreover, the different influence on binding of these two ligands by mutations located deep along the inner side of TM6 (Phe270Ala, Tyr266Ala, Trp263Ala) indicates a nonequivalent positioning, although occupying the same binding crevice. Furthermore, binding data indicate the Ile202Phe mutation, which mimics the wild-type rat NK_2 receptor sequence, as a species selectivity determinant. In summary, data with mutant receptors describe, for these new tachykinin NK_2 receptor antagonists, a binding site which is partially overlapping either with that of the cyclized peptide antagonist nepadutant (cyclo-{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dpr-Leu]cyclo(2β-5β)} or the nonpeptide antagonist SR48968 ((S)-*N*-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide).

Keywords: Binding site; G protein-coupled receptor; Molecular modelling; Nonpeptide antagonists; Site-directed mutagenesis

1. Introduction

Neurokinin A (NKA, His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH2) is a tachykinin peptide which displays the highest affinity for the tachykinin NK2 receptor, a G-protein coupled with seven helices spanning through the cell membrane, which mediates its biological effects (Gerard et al., 1990; Severini et al., 2002).

Antagonists for the peripheral tachykinin NK2 receptors are considered as potential innovating therapies in various diseases, such as neurogenic bladder hyperreflexia and irritable bowel syndrome (Lecci and Maggi, 2003).

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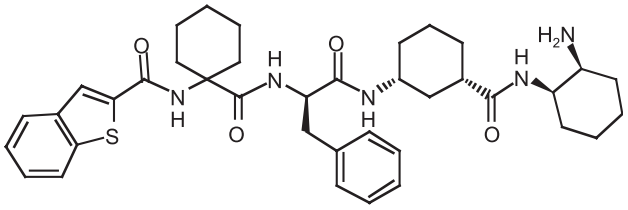
MEN 13918



MEN 14268

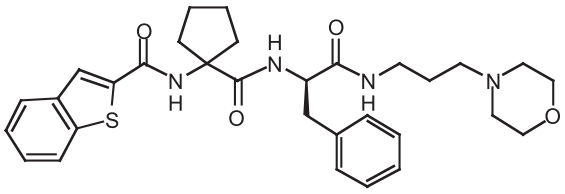


Fig. 1. Structures of MEN13918 (Nγ{Nα[Nα(benzo[*b*]thiophen-2-yl)carbonyl]-1-aminocyclohexan-1-carboxy]-D-phenylalanyl}-3-*cis*-aminocyclohexan-1-carboxylic-acid-*N*-(1*S*,2*R*)-2-aminocyclohexyl)amide trifluoroacetate salt) and MEN 14268 (Nα[Nα(benzo[*b*]thiophen-2-yl)-carbonyl)-1-aminocyclopentane-1-carboxyl]-D-phenylalanine-*N*-[3(morpholin-4-yl)propyl]amide trifluoroacetate salt).

We previously constructed a three-dimensional receptor model of the tachykinin NK_2 receptor and, by means of site-directed mutagenesis, have identified a subset of amino acids that participate in the binding site of peptide and nonpeptide antagonist ligands (Giolitti et al., 2000, 2002).

The aim of the present study was the investigation of the binding interaction mode of a new class of tachykinin NK_2 receptor antagonists, such as MEN13918 (Nγ{Nα[Nα(benzo[*b*]thiophen-2-yl)carbonyl]-1-aminocyclohexan-1-carboxy]-D-phenylalanyl}-3-*cis*-aminocyclohexan-1-carboxylic-acid-*N*-(1*S*,2*R*)-2-aminocyclohexyl)amide trifluoroacetate salt) and MEN14268 (Nα[Nα(benzo[*b*]thiophen-2-yl)carbonyl)-1-aminocyclopentane-1-carboxyl]-D-phenylalanine-*N*-[3(morpholin-4-yl)propyl]amide trifluoroacetate salt) (Fig. 1), here termed as linear pseudopeptides. By means of data obtained using site-directed mutagenesis at the human tachykinin NK_2 receptor and binding competition experiments, a molecular model of MEN13918 and MEN14268 docked to their recognition site in the human tachykinin NK_2 receptor is presented.

2. Materials and methods

2.1. Materials

[^{125}I]NKA (specific activity 2000 Ci mmol^{-1}) was provided by Amersham, [^{3}H]SR48968 (specific activity 25.5 Ci mmol^{-1}) (Emonds-Alt et al., 1993) by Perkin Elmer New England Nuclear, and [^{3}H]nepadutant (specific activity 30 Ci mmol^{-1}) (MEN11420; (cyclo-{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dpr-Leu]cyclo(2β–5β)}; Renzetti et al., 1998) was synthesized by SibTech (Elmsford, NY). Peptides were obtained from Neosystem. All salts used were purchased from Merck and all other materials from Sigma. NK_2 receptor antagonists were synthesized in Menarini Ricerche, and SR48968 ((*S*)-*N*-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) was a kind gift of Sanofi. Nonpeptide ligands were dissolved in dimethylsulphoxide up to 100 μM. All compounds were stored at −25 °C.

2.2. Organ bath experiments

Male albino rats (Wistar strain, 275–350 g) and male albino guinea-pigs (250–300 g) were decapitated under ether anaesthesia. Guinea-pig ileum longitudinal muscle myenteric plexus strips, rat urinary bladder longitudinal detrusor muscle strips, and guinea-pig colon circular muscle strips deprived of the mucosa were prepared as described previously (Catalioto et al., 1998; Santicioli et al., 1997) Mucosa-free strips of human detrusor muscle were excised from the urinary bladder dome of patients undergoing cystectomy because of carcinoma of the bladder base, and set up as described previously (Patacchini et al., 2000). All preparations were placed in 5-ml organ baths filled with oxygenated (96% O_2 and 4% CO_2) Krebs solution, having the following composition: NaCl, 119 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.5 mM; $CaCl_2$, 2.5 mM; KCl, 4.7 mM and glucose 11 mM.

The motor activity of guinea-pig ileum longitudinal muscle preparation (load 3 mN), guinea-pig colon circular muscle preparation (load 10 mN), rat urinary bladder longitudinal detrusor muscle (load 5 mN) was recorded isotonically, while that of human detrusor muscle (load 10 mN) isometrically. The activity of MEN13918 and MEN14268 was evaluated in the various preparations as the ability to block agonist-induced contractile responses.

Approval of the experimental protocols with human tissues and with animals was obtained from the local Ethics committee.

2.3. Site-directed mutagenesis of human NK_2 receptor cDNA

Site-directed mutagenesis of the NK_2 receptor cDNA was performed by the phosphorothioate technique of Eckstein using single-stranded DNA of the NK_2 receptor cDNA in pBlueScript II SK(−), and an in vitro mutagenesis kit (Sculptor™, Amersham). Wild-type and mutated cDNAs were cloned in pmCMVβSV1dhfr, under the transcriptional control of the murine cytomegalovirus major immediate early promoter (Rotondaro et al., 1997). The complete coding sequence of cDNAs for wild-type and mutated tachykinin NK_2 receptors was confirmed by DNA sequencing.

2.4. CHO cell expression

Large-scale preparation of vector DNA for transfection experiments was carried out using a Qiagen maxi-preparation column (Qiagen, Hilden, Germany). Wild-type and mutated NK_2 receptor cDNAs in pmCMVβSV1dhfr were introduced by lipofection into the dihydrofolate reductase-(DHFR)-deficient CHO cell line CHO DUKX-B11. Stable $DHFR^+$ transformants were selected into nucleoside-free α-modification essential Eagle' s medium containing 10% dialyzed foetal calf serum and 2 mM L-glutamine; 12–14 days after transfection, more than 100 individual $DHFR^+$ clones were pooled and grown to mass culture. The cells were subcultured by using 0.25% trypsin and 1 mM ethylenediaminetetraacetate to detach them, and then cultured in 175-cm^2 flasks and maintained in a humidified atmosphere at 37 °C with 5% CO_2.

2.5. Membrane preparation

Cells at confluence were rinsed with ice-cold phosphate buffered saline without Ca^{2+} and Mg^{2+} and pelleted by centrifugation at 200 × *g*, 10 min at 4 °C. The pellet was homogenized with a Polytron (PT 3000, Kinematica) at 15,000 r.p.m. for 30 s in 30 ml of 50 mM Tris–HCl, pH 7.4, containing bacitracin (0.1 mg/ml), chymostatin (0.01 mg/ml), leupeptin (5 μg/ml) and thiorphan (10 μM) (buffer A). The homogenate was centrifuged at 25,000 × *g* for 1 h at 4 °C and the pellet was resuspended in the binding buffer composed of buffer A supplemented with 150 mM NaCl, 5 mM $MnCl_2$, and 0.1% bovine serum albumin to obtain 5 mg ml^{-1} membrane 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 and mixed to give a homogeneous membrane suspension.

2.6. Radioligand binding

Binding assay was performed at room temperature in a final volume of 0.5 ml, and an incubation time according to the used radioligand: 30 min was used for [^{125}I]NKA and [^{3}H]SR48968, and 60 min for [^{3}H]nepadutant. Each radioligand was used at a concentration which was comparable to or less than the calculated K_d value ([^{125}I]NKA 0.2 nM, [^{3}H]SR48968 0.2 nM, and [^{3}H]nepadutant 0.4 nM) giving a bound less than 10% of the total added radioligand concentration, and a specific binding representing approximately 70–80% of the total binding. Nonspecific binding was defined as the amount of labeled ligand bound in the presence of the appropriate unlabeled ligand (NKA, SR48968, or nepadutant, 1 μM). Competing ligands were tested in a wide range of concentrations (1 pM–1 μM). Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard Instrument), pre-soaked for at least 2 h in polyethylenimine 0.3%, and using a MicroMate 96 Cell Harvester (Packard Instrument). The tubes and filters were then washed five times with 0.5-ml aliquots of Tris buffer (50 mM, pH 7.4, 4 °C). Filters were dried and soaked in Microscint 40 (50 μl/well; Packard Instrument), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument).

2.7. Data analysis

All values in the text, tables or figures are mean and 95% confidence limits (c.l.), or mean ± S.E.M. of the given number of experiments.

In functional experiments (smooth muscle contractility) responses to NKA either in the absence or presence of antagonist were normalized towards the maximal effect of control NKA. Concentration–response curves were analyzed by fitting the data with the GraphPad Prism program (San Diego, CA, USA) in order to determine the molar concentration of the agonist producing the 50% of its maximal effect.

The apparent affinity of antagonists was expressed as apparent pK_B calculated from the equation: $pK_B = \log [CR - 1] - \log$ [antagonist concentration], where CR is the ratio of equiactive concentrations of agonist in the presence and absence of antagonist (Kenakin, 1997).

Binding data were fitted by nonlinear regression using GraphPad Prism 3.0 in order to determine the equilibrium dissociation constant (K_d) from homologous competition experiments, and the ligand concentration inhibiting the radioligand binding of the 50% (IC_{50}) from heterologous competition experiments. K_i values were calculated from IC_{50} using the Cheng–Prusoff equation ($K_i = IC_{50}/(1+$ [radioligand]$/K_d$) according to the concentration and K_d of the used radioligand at each mutant receptor.

2.8. Molecular modelling of receptor and antagonist ligands

The three-dimensional model for the transmembrane region of the human tachykinin NK_2 receptor has been obtained starting from the crystal structure of bovine rhodopsin (Palczewski et al., 2000), as already described (Giolitti et al., 2002). Amino acid side chains were optimized for contacts on a SGI "O2" Workstation running Sybyl molecular modelling software (Tripos, St. Louis, MO, USA). MEN13918 and MEN14268 conformations were obtained from simulated annealing conformational searches in Sybyl, and comparing the lowest energy conformers with crystal structures of the relevant fragments (Cambridge Crystallographic Database), to select the best conformer.

3. Results

3.1. Antagonist activity of MEN13918 and MEN14268 in various smooth muscle contractility assays for tachykinin receptors

Neither MEN13918 nor MEN14268 produced agonist effects, up to 1 μM concentrations, in any of the smooth muscle preparations tested.

Both compounds potently antagonized NK_2 receptor-mediated contractions produced by NKA in the human isolated urinary bladder (pK_B 9.1 and 8.3 for MEN13918 and MEN14268, respectively; Table 1). In the guinea pig colon preparation both MEN13918 and MEN14268 antagonized the contractile effects produced by the NK_2 receptor agonist [βAla^8]-NKA(4–10), without depressing its maximal effect. Their apparent affinity (pK_B) was 9.2 and 7.7, respectively (Table 1). In contrast, in the rat urinary bladder both compounds were barely effective, up to micromolar concentrations, in blocking NK_2 receptor-mediated contractions elicited by [βAla^8]-NKA(4–10) ($pK_B = 6.8$ and < 6 for MEN13918 and MEN14268, respectively; Table 1).

Neither MEN13918 nor MEN14268 affected NK_1 or NK_3 receptor-mediated contractions in the guinea pig ileum, induced by [Sar^9]-substance P sulfone or senktide, respectively ($pK_B < 6$; Table 1).

3.2. MEN13918 and MEN14268 affinity measured at binding site of radiolabeled agonist and antagonists to the wild-type and mutant human NK_2 receptors

Competition binding experiments were carried out at the human tachykinin NK_2 receptor expressed in CHO cells.

Table 1
Antagonist activity of MEN13918 and MEN14268 in various smooth muscle contractility assays for tachykinin receptors

Assay	$pK_B \pm$ S.E.M.	
	MEN13918	MEN14268
NK_1 receptor		
Guinea pig ileum	< 6	< 6
NK_2 receptor		
Guinea pig colon	9.2 ± 0.1	7.7 ± 0.1
Rat urinary bladder	6.8 ± 0.2	< 6
Human urinary bladder	9.1 ± 0.2	8.3 ± 0.2
NK_3 receptor		
Guinea pig ileum	< 6	< 6

Concentration–response curves to the receptor selective agonist (see Materials and methods) were performed in the absence and in the presence of the antagonist, added with a preincubation period of 15 min. The curves were fitted to obtain the respective EC_{50} values and the apparent affinity of competitive antagonists was expressed in terms of pK_B calculated from the equation: $pK_B = \log[CR - 1] - \log$ [antagonist concentration] where CR is the ratio of equiactive concentrations of agonist in the presence and absence of antagonist. pK_B is shown as the mean ± S.E.M. All values are obtained from four to eight experiments.

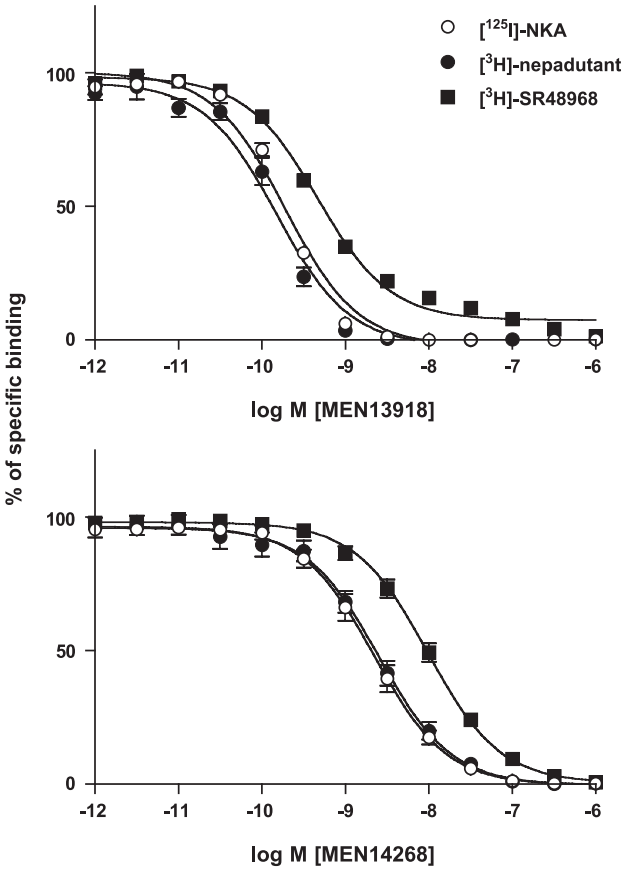


Fig. 2. Comparison of binding profile of MEN13918 and MEN 14268 at the wild-type human tachykinin NK2 receptor. Heterologous competition binding curves for antagonist ligands MEN13918 and MEN 14268 were performed in membrane preparations from stably transfected CHO cells expressing the human tachykinin NK_2 receptor, using [^{125}I]NKA, [^{3}H]nepadutant, and [^{3}H]SR48968 as radioligands. Experimental conditions are described in Material and methods.

The affinity of MEN13918 and MEN14268 in inhibiting the binding of different radioligands was in the subnanomolar and nanomolar concentration range, respectively (Fig. 2), the affinity of MEN13918 being from 10- to 20-fold higher than that of MEN14268 (Table 2).

A total of 10 mutant receptors were investigated, all located in the transmembrane (TM) portion of the tachykinin NK_2 receptor sequence, with the exception of Cys281-Tyr (C281Y) in the fourth extracellular loop. Investigated receptor residues in the TM portion are schematically represented in Fig. 3. The affinity of MEN13918 and MEN14268 was evaluated in inhibiting the binding of [^{125}I]NKA, [^{3}H]nepadutant, and [^{3}H]SR48968, and compared to that of the unlabeled ligands.

The Thr171Ala (T171A) mutant receptor did abrogate the [^{3}H]nepadutant binding only, as previously reported (Giolitti et al., 2002), whereas leaving unaffected the affinity constants (K_d) of [^{125}I]NKA and [^{3}H]SR48968. The ability of MEN13918 and MEN14268 to compete for these radioligands was not affected by the T171A mutation.

Table 2
Binding affinity of MEN13918 and MEN14268 at wild-type and mutant human tachykinin NK_2 receptors

	[^{125}I]-NKA					[^{3}H]-Nepadutant					[^{3}H]-SR48968				
	NKA	MEN13918		MEN14268		Nepadutant	MEN13918		MEN14268		SR48968	MEN13918		MEN14268	
	K_d	K_i	F_{mut}	K_i	F_{mut}	K_d	K_i	F_{mut}	K_i	F_{mut}	K_d	K_i	F_{mut}	K_i	F_{mut}
Wild type	4.7 (3.1– 6.3)	0.2 (0.1–0.3)		2.8 (2.3–3.3)		2.2 (1.7–2.8)	0.1 (0.1–0.2)		2.2 (1.7–2.7)		0.8 (0.6–0.9)	0.4 (0.3–0.5)		6.9 (5.5–8.3)	
C167G	n.d.b.					n.d.b.					0.9 (0.1–8.6)	n.i.		n.i	
T171A	3.4 (2.3–4.4)	0.1 (0.06–0.15)	0.5	5.1 (4.1– 6.0)	1.8	n.d.b.					0.8 (0.7–0.9)	0.4 (0.2–0.6)	1.0	16 (12.3–19.6)	2.3
I202F	3.8 (2.2–6.5)	4.7 (3.5– 5.8)	**24**	477 (323– 631)	**170**	0.5 (0.3–0.8)	5.7 (5.3–6.1)	**57**	>1000	**>500**	1.0 (0.8–1.2)	14.5 (12.2–16.8)	**36**	>1000	**>150**
Y206A	n.d.b.					n.d.b.					3.7 (2.5–6.3)	13.0 (4.5–37.5)	**33**	12.2 (2.1–29.3)	1.8
W263A	n.d.b.					n.d.b.					3.1 (1.7–4.6)	6.8 (5.5–8.1)	**17.0**	22.1 (7.4–36.9)	**3.2**
Y266F	4.6 (3.8–6.5)	7.1 (1.5–12.6)	**36**	104 (74.4–133)	**37**	n.d.b.					1.4 (1.2–1.6)	8.5 (2.3–14.6)	**21**	93.9 (47.2–141)	**14**
F270A	n.d.b.					n.d.b.					4.7 (2.2–7.1)	80.6 (23.9–137)	**202**	148 (71.1–306)	**21**
C281Y	5.9 (3.9–7.9)	0.2 (0.1–0.3)	1	2.5 (0.4–4.6)	0.9	2.5 (2.0–3.2)	0.1 (0.1–0.2)	1.0	0.8 (0.6–0.9)	0.4	0.7 (0.5–0.9)	0.8 (0.7–0.9)	2.0	9.8 (3.9–15.8)	1.4
Y289F	3.2 (2.5–3.2)	1.9 (1.8–2.0)	**9.5**	200 (193–207)	**71**	7.9 (6.1–10.4)	2.4 (1.9–3.1)	**24**	291 (191–443)	**132**	n.d.b.				
Y289T	n.d.b.					29.5 (20.2–39.8)	117 (113–120)	**1170**	95.2 (81.1–109)	**43**	n.d.b.				

Ki values of competing ligands were calculated from heterologous inhibition experiments according to the concentration and Kd of the used radioligand for each mutant receptor (See Methods), and is expressed in nanomolar concentration. Fmut is calculated as Ki (mutant receptor)/Ki (wild type human NK2 receptor), and corresponds to fold decrease in affinity. Significant Fmut values (> 3) are indicated in bold. Values are mean and in 95% confidence limits in parenthesis of at least 3 experiments, each one performed in duplicate. n.d.b. not detectable binding. n.i. no inhibition up to 1 μM competing ligand concentration.

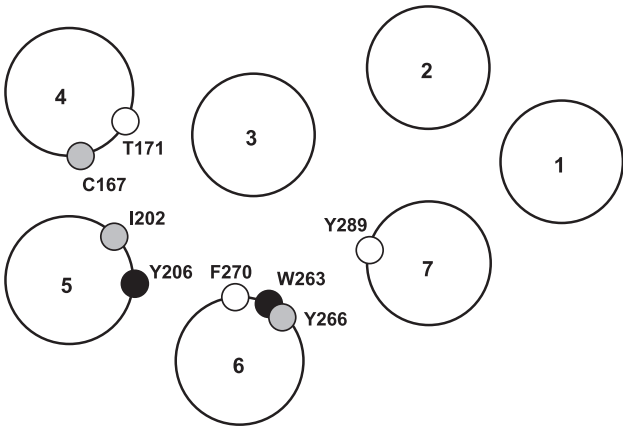


Fig. 3. Cross-sectional schematic sight of the seven transmembrane domains of the human tachykinin NK2 receptor. The transmembrane domains (large circles 1 to 7) bear the investigated aminoacid residues (small circles). Aminoacid residues which lie at the same level in the helix are filled with the same colour, the white ones being in the outer portion and the darkest being deeper in the membrane. C167G, Y206A, W263A, F270A, and Y289T are the mutations which determine a loss of affinity for the natural agonist NKA (results in Table 2). C167G, T171A, Y206A, W263A, Y266F, F270A are the mutations which abrogate the binding of the cyclic peptide antagonist nepadutant (its affinity is reduced by the Y289F and Y289T mutations by 4- and 13-fold, respectively). The binding of the nonpeptide antagonist SR48968 is abolished by the Y289F and Y289T mutations only, whereas its affinity is reduced at the W263A, Y266F, F270A (by 4-, 2-, and 6-fold, respectively).

Cys167 residue in the human NK_2 receptor sequence corresponds to a Gly residue in the human NK_1 receptor sequence (Takahashi et al., 1992). The Cys167Gly (C167G, TM4) mutation did abolish the binding of [^{125}I]NKA, [^{3}H]nepadutant, and drastically reduced the binding of [^{3}H]SR48968 radioligand. MEN13918 and MEN14268 were not able to inhibit at any extent the binding of [^{3}H]SR48968 up to 1 μM concentration.

Ile202Phe (I202F) and C281Y are mutations that spontaneously occur in the rat and hamster NK_2 receptor sequence (Sasai and Nakanishi, 1989; Aharony et al., 1994): no differences were observed at these mutant receptors with all the tested radioligands. Similarly to NKA, nepadutant and SR48968, MEN13918 and MEN14268 affinity was not affected by the C281Y mutation. On the contrary, the affinities of MEN13918 and MEN14268 were significantly reduced, by 20–60-fold and >150-fold, respectively, by the I202F mutation, whichever was the used radioligand.

The Tyr206Ala (Y206A) mutation, while completely abolished the binding of the agonist [^{125}I]NKA and the antagonist [^{3}H]nepadutant radioligands, reduced by only 4.6-fold the affinity of the nonpeptide antagonist [^{3}H]SR48968, as determined by homologous displacement curve. In agreement with radioligand experiments, NKA or nepadutant was not able to inhibit at any extent the [^{3}H]SR48968 binding, up to micromolar concentration (data not shown). The affinity of the MEN13918 ligand resulted significantly decreased (32-fold) in inhibiting of [^{3}H]SR48968 binding, whereas that of MEN14268 was unaffected by this mutation. On the other hand, none of these ligands was able to displace all the [^{3}H]SR48968 bound at the Y206A mutant, the maximal inhibition being $60 \pm 7\%$.

The three mutated residues belonging to TM6 were localized as facing in the inner receptor crevice: Trp263Ala (W263A) deeper, and Tyr266Phe (Y266F) and Phe270Ala (F270A) closer to membrane surface, and all of them did abrogate the binding of the antagonist radioligand [^{3}H]nepadutant, whereas [^{125}I]NKA was able to bind only at the Y266F mutation but not at the W263A or the F270A mutants. The binding of [^{3}H]SR48968 was only weakly (2–6-fold) affected by TM6 mutations. Inhibition curves with linear pseudopeptide ligands at the [^{3}H]SR48968 binding highlighted a different pattern. The W263A mutation did impair the MEN13918 affinity by 17-fold, and that of MEN14268 by 3-fold. The Y266F mutation similarly reduced the affinity of both ligands at the [^{3}H]SR48968 (14–21-fold) and [^{125}I]NKA binding (36–37-fold). Lastly, the F270A mutation drastically diminished the affinity of MEN13918 (202-fold), and in a lesser extent that of MEN14268 (20-fold).

The affinity of the two linear pseudopeptides was evaluated at the only mutated residue which crucially abolished the nonpeptide antagonist [^{3}H]SR48968 binding. Tyr289-Phe (Y289F) and Tyr289Thr (Y289T) mutations produced a drastic decrease in the affinity of both MEN13918 and MEN14268. The Y289F mutation affected at a greater extent the affinity of MEN14268 (71–132-fold) as compared to that of MEN13918 (9–24-fold), at both [^{125}I]NKA and [^{3}H]nepadutant binding site. On the contrary, the Y289T mutation changed the rank order of the affinity measured at the [^{3}H]nepadutant binding site, the MEN13918 being drastically affected (>1000-fold decrease in K_i) as compared to MEN14268 (43-fold).

4. Discussion

In the field of tachykinin NK_2 receptor antagonists we previously reported the pharmacological profile of bycyclic peptide antagonists such as MEN10,627 (cyclo-(Met-Asp-Trp-Phe-Dap-Leu]cyclo(2β-5β)}; Maggi et al., 1994) and nepadutant (Catalioto et al., 1998). These structures were further simplified leading to the monocyclic peptide MEN 11558 (cyclo-{-Suc-Trp-Phe-[(*R*)-NH-CH(CH2-C6H5)-CH2-NH]}; Giannotti et al., 2000), and by combining rational design and site-directed mutagenesis at the human tachykinin NK_2 receptor we described their binding site (Giolitti et al., 2000, 2002). In the present paper the affinity of MEN13918 and MEN14268 has been compared at the wild-type and point-mutated tachykinin NK_2 receptors, and experimental data used to refine on the modelling of the antagonist–receptor complex. The bulk of experimental data indicate that the binding site of the two linear pseudo-

peptide ligands is in part overlapping to that of the agonist NKA or antagonist nepadutant, comprised amongst TM4, TM5, TM6 (as characterized by heterologous binding experiments at [^{3}H]SR48968 binding site), besides sharing a critical determinant for binding of the nonpeptide SR48968 in TM7 (as characterized by heterologous binding experiments at [^{3}H]nepadutant binding site).

Structurally, MEN13918 and MEN14268 share some chemical moieties, such as the benzothiophenyl group and the stereochemistry of the phenylalanine residue (D-Phe) present in their structure. On the other hand, a minor difference is represented by the cyclohexyl moiety in the MEN13918 structure, and the cyclopentyl moiety in that of MEN14268, but the greatest difference is in the aminic portion of the two ligands (Fig. 1), to which we attribute the difference in receptor affinity and antagonist potency. Indeed, the mutational analysis revealed that they share a common binding pocket at the level of receptor transmembrane portion, although having a different positioning and interaction mode with some receptor residues, as described in Fig. 4. This concept is highlighted by the Y289 mutant receptors. Both the hydroxylic group and the aromatic ring of the Y289 residue, located in the upper portion of TM7, have been shown to be relevant for the affinity of the nonpeptide NK_2 receptor antagonist SR48968 (Huang et al., 1995; Renzetti et al., 1999). Moreover, the substitution of the Y289 residue with a T, which conserves only the hydroxylic group, was able to restore the binding of nepadutant only (Giolitti et al., 2000). The affinities of MEN13918 and MEN14268 appear as differently biased by the two substitutions at the Y289 residue, the Y289F mutation preferably unfavouring the high affinity interaction with MEN14268, whereas the Y289T mutation is detrimental for that of MEN13918. These data would favour an interaction of the basic groups of the ligands with the Y289 residue, and a stabilization of the MEN13918 receptor interaction by means of the cyclohexyl, bearing the amine group, with the aromatic group of Y289 (Fig. 4).

C167, located in TM4 of the human NK_2 receptor sequence, corresponds to a G residue in the human NK_1 receptor sequence (Takahashi et al., 1992), which is responsible for tachykinin receptor subtype affinity and selectivity (Ciucci et al., 1997). The C167G mutation does abolish the binding of the antagonist nepadutant, besides that of the peptide agonist NKA, and reduced the measured binding with the SR48968 radioligand leaving unaltered its affinity. The reduction in receptor density by this mutant could be a consequence of a hampered receptor expression/maturation or a change in the distribution of its conformational states. Nevertheless, this mutant appears to affect the binding of the linear pseudopeptides: the Cys residue has a strong lipophilic character which matches well with that of the benzothiophene ring in MEN13918 and MEN14268 (Fig. 4).

In TM6 three mutants were studied, each one distant from the other by about one helical turn. As already observed for the endogenous ligand NKA (Huang et al., 1995), F270A is the mutation which impaired the binding of MEN13918 at the greatest extent (200-fold, MEN14268 by 20-fold). On the other hand, deeper mutations in TM6 reduced the affinity of both pseudopeptide ligands but in a reduced manner, in any case MEN13918 being more affected than MEN14268. These data, together with the different effect of the Y206A (TM5) mutant on the binding of MEN13918 and MEN14268, suggest a nonequivalent positioning of the cyclohexane, or cyclopentane, and D-phenylalanine moieties of the two ligands which lie at different levels (Fig. 4).

Overall, these findings support the concept that a common binding site for small ligands is conserved within the TM domain of all G protein-coupled receptors (GPCRs) regardless of the native ligand (Schwartz, 1994). Moreover, it is worth mentioning that in the upper part of TM6, a phenylalanine residue has been reported as a common interaction point for peptide and nonpeptide ligands for other peptide GPCRs, and a series of hydrophobic residues facing in the same receptor crevice, where the TM3, TM6, and TM7 interface, is quite conserved amongst GPCRs (Huang et al., 1994; Lundstrom et al., 1997; Turcatti et al., 1997; Meini et al., 2002; Bellucci et al., 2003; Meini et al., 2004).

Several evidences have been presented in the past indicating a pharmacological heterogeneity for the tachykinin NK_2 receptor. These differences were highlighted by the opposite rank order of potencies obtained with linear and cyclic peptide antagonists (Maggi et al., 1990; Ireland et al., 1991) and the agonist/antagonist profile of a pseudopeptide molecule (MDL28,564; Maggi et al., 1992) in bioassays for the NK_2 receptor variants in rabbit, guinea pig, human, bovine species on one hand versus hamster and rat species on the other (see Maggi, 1994 for review). Generally, the pharmacological and sequence differences in cloned receptors between species have facilitated the identification of nonconserved amino acids as critical structural determinants of ligands binding, but this is not the case for the NK_2 receptor. We present evidence that both MEN13918 and MEN14268 display a low (μM) apparent affinity at the rat NK_2 receptor despite their high affinity determined both in binding and in functional studies at the human NK_2 receptor. Results obtained at the I202F mutant NK_2 receptor, located in the upper part of the TM5, which spontaneously occurs in the rat tachykinin NK_2 receptor, suggest that this residue could be largely responsible for the low affinity pharmacological profile of these ligands (MEN13918 and MEN14268) observed in the rat species. Moreover, the observed increase of nepadutant affinity for the same mutant is consistent with previous findings indicating a higher antagonist potency in rat (pK_B 9.0) versus human (pK_B 8.4) detrusor smooth muscle preparations (Catalioto et al., 1998; Patacchini et al., 2000). The I202 present in the human and guinea pig species would stabilize the interaction with the cyclohexyl or cyclopentyl moieties of MEN13918 and MEN14268, respectively (Fig. 4), where-

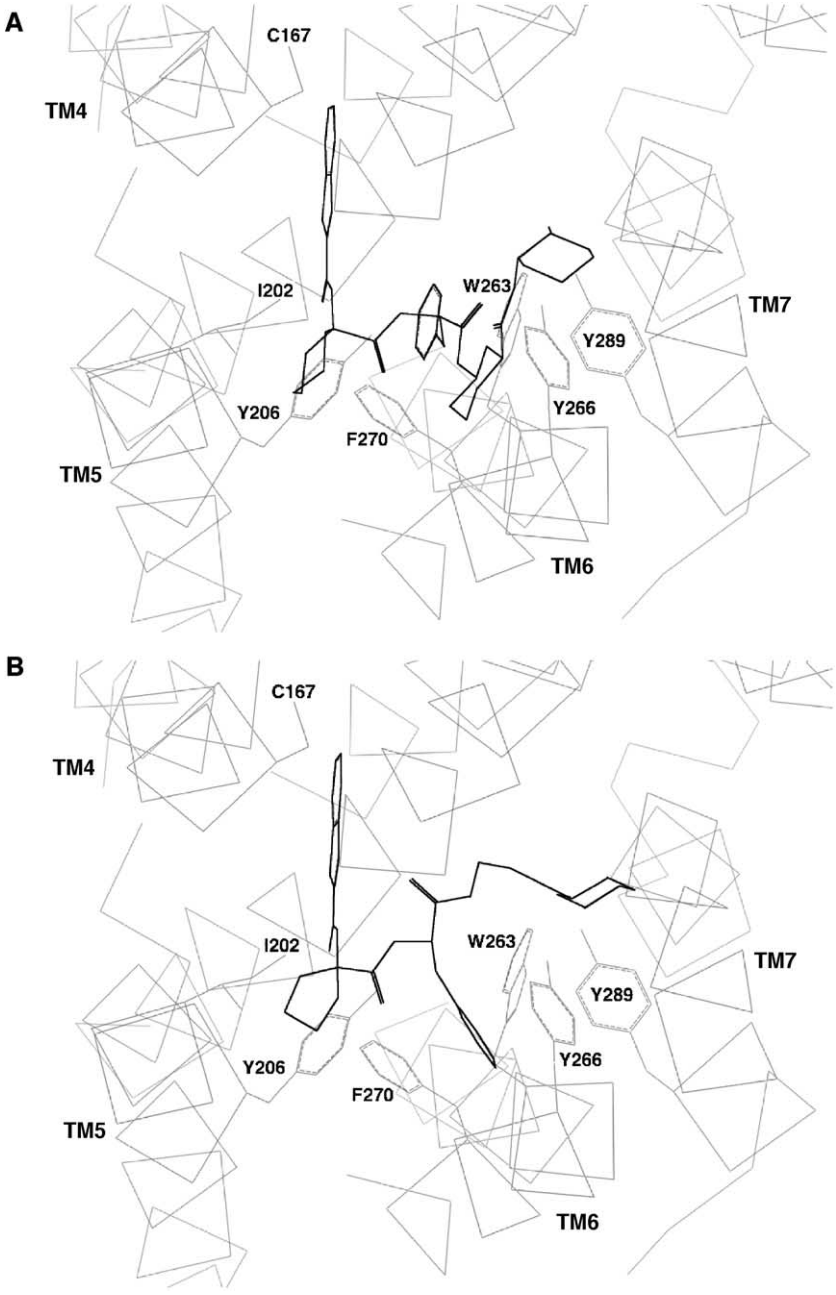


Fig. 4. Model of MEN13918 and MEN14268 docked to their recognition site in the wild-type human tachykinin NK2 receptor. The models of MEN13918 (A) and MEN14268 (B) are viewed from the extracellular side in the transmembrane pocket of the human tachykinin NK2 receptor. In the suggested binding modes the basic groups of the ligands would interact with the Y289 residue in the upper part of TM7: MEN13918 primary amine (and possibly the close amidic function) and the tertiary amine of the morpholine ring of MEN14268 would interact with the hydroxyl group of Y289. The terminal cyclohexyl ring of MEN13918 appears to interact with the aromatic ring of Y289. The benzothiophenyl ring of both ligands would interact with the C167 residue in TM4. Both the cyclohexyl moiety in the MEN13918 structure and the cyclopentyl moiety in that of MEN14268 would interact with I202 in TM5 and F270 in TM6. A further difference in the two binding modes is relative to the Phenylalanine ring: for MEN13918 an interaction with the aromatic rings of Y206 and W263 is proposed, while in the case of MEN14268 with the aromatic rings of F270 and Y266.

as the presence of a bulky aromatic residue in the receptor sequence of the rat species (F202) may hinder the proper interaction to form a high affinity ligand receptor complex.

As a whole, the present study is part of a process for the optimization of pseudopeptide antagonists for the human tachykinin NK_2 receptor, whose realization has exploited the information obtained by combining mutagenesis and binding studies with modelling and rational design (Giolitti et al., 2000, 2002; the present study), the goal being MEN13918. A binding site, which is partially overlapping either with that of the cyclized peptide antagonist nepadutant and that of the nonpeptide antagonist SR48968, is described for these new NK_2 receptor antagonist ligands.

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