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Pharmacology of an original and selective nonpeptide antagonist ligand for the human tachykinin NK_2 receptor

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Abstract

The pharmacological outline of a novel and original antagonist at the human tachykinin NK_2 receptor is presented, namely MEN13510 (*N*-*N*′-bis-[2-(1*H*-indol-3-yl)-ethyl]-*N*,*N*′-bis-(3-thiomorpholin-4-yl-propyl)-phthalamide). MEN13510 retained nanomolar affinity for the human tachykinin NK_2 receptor (K_i 6.4 nM), and micromolar affinity for the human tachykinin NK_1 and NK_3 receptors. A competitive antagonism is indicated by the Schild analysis (pK_B 7.8, slope −0.94) of concentration–response curves of NKA induced inositolphosphates accumulation in Chinese hamster ovary (CHO) cells expressing the human NK_2 receptor in the presence of MEN13510 (30–300 nM concentration range). The MEN13510 interaction with the human NK_2 receptor was evaluated by means of heterologous inhibition binding experiments, by using agonist and antagonist radioligands ([^{125}I]NKA, [^{3}H]nepadutant, [^{3}H]saredutant) at a series of mutant receptors having single aminoacidic substitutions of residues located in transmembrane (TM) segments 3, 4, 5, 6, and 7. MEN13510 affinity was not affected by the mutations in TM 3 and 4 (Q109A, F112A, T171A, C167G), and it was reduced by 10-fold at the I202F mutant, but not at the Y206A (TM4). Amongst the investigated mutants bearing the mutated residues in TM6 (F270A, Y266F, W263A) only F270A decreased the MEN13510 affinity by 7-fold. Even mutations in TM7 did reduce MEN13510 affinity by 32-fold (Y289T, but not Y289F) and 13-fold (F293A). Studied mutations represent the human tachykinin NK_2 receptor discriminants involved in the binding of previously reported peptidic and nonpeptidic antagonists, against which results obtained with MEN13510 are compared. Results indicate that the binding site of this antagonist is, at least in part, overlapping to that described for NKA or saredutant. Finally we show that MEN13510 retains nanomolar affinity for the recently discovered splice variant of the human tachykinin NK_2 receptor, namely β isoform, as it has been described for the nonpeptide antagonist saredutant.

Keywords: Binding site; G protein-coupled receptor; Nonpeptide antagonist; Site-directed mutagenesis; Tachykinin NK_2 receptor β isoform

1. Introduction

Tachykinins, such as substance P (SP, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), neurokinin A (NKA, H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2), and neurokinin B (NKB, H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2), are a family of small peptides that share a common C-terminal sequence, and are mainly located in the peripheral endings of capsaicin-sensitive primary afferent neurons innervating the airways and the urinary tract, as well in intrinsic neurons of the gut (Maggi et al., 1993). Tachykinins exert their biological activity by acting at three distinct receptors although with a different rank order of potency, such as SP, NKA, and NKB are

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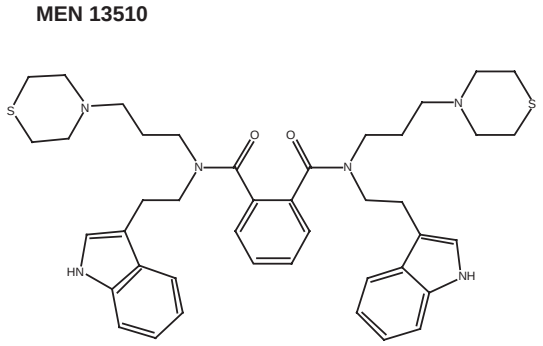

Fig. 1. Structure of MEN13510 (*N*-*N*′-bis-[2-(1*H*-indol-3-yl)-ethyl]-*N*,*N*′-bis-(3-thiomorpholin-4-yl-propyl)-phthalamide).

preferring ligands for the tachykinin NK_1, NK_2, and NK_3 receptors, respectively. Tachykinin receptors have been sequenced and shown to belong to the superfamily of G-protein-coupled seven transmembrane spanning receptors (Gerard et al., 1990, 1991; Buell et al., 1992).

Antagonists for the peripheral tachykinin NK_2 receptors are currently considered as potential innovating therapies in various diseases, such as neurogenic bladder hyperreflexia, irritable bowel syndrome (Lecci and Maggi, 2003; Lecci et al., 2004), and airways diseases (Joos and Pauwels, 2001).

In the field of tachykinin NK_2 receptor antagonists we have previously discussed the progressive construction of molecules going from peptide to pseudopeptide structures (Meini et al., 2004). This path exploited the peptide cyclization process (Pavone et al., 1995) with several simplification steps (MEN11558, Giannotti et al., 2000; Giolitti et al., 2002) which led to the discovery of a series of nonpeptide antagonist ligands (Altamura et al., 2002) whose structure is quite original amongst the known tachykinin NK_2 receptor antagonists (Fattori et al., 2004). The best compound in this series is MEN13510 (*N*-*N*′-bis-[2-(1*H*-indol-3-yl)-ethyl]-*N*,*N*′-bis-(3-thiomorpholin-4-yl-propyl)-phthalamide, Fig. 1, compound 6 in Altamura et al., 2002).

The present study aims to pharmacologically characterize MEN13510 in terms of tachykinin receptors selectivity and antagonism. Moreover, we present data on the MEN13510 affinity at 13 mutated human tachykinin NK_2 receptors by exploiting three different radioligands for which different receptor binding sites were previously modelled (Giolitti et al., 2000)., i.e. NKA and the antagonists nepadutant (Catalioto et al., 1998) and saredutant (SR48968, Emonds-Alt et al., 1993). Finally the MEN13510 affinity for the recently identified splice variant of the tachykinin NK_2 receptor is shown (Candenas et al., 2002).

2. Materials and methods

2.1. Materials

[^{3}H]SP (specific activity 41 Ci/mmol) and [^{125}I]NKA (specific activity 2000 Ci/mmol) were provided by Amersham Biosciences (Buckinghamshires, UK), [^{3}H]saredutant (specific activity 25.5 Ci/mmol) (SR48968, Emonds-Alt et al., 1993), [^{125}I]MePhe^{7}NKB (specific activity 2000 Ci/mmol) and *myo*-[1,2-^{3}H] inositol (specific activity 75 Ci/mmol) by Perkin Elmer New England Nuclear (Boston, MA, USA), and [^{3}H]nepadutant (specific activity 30 Ci/mmol) (MEN11420; (cyclo-{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dpr-Leu]cyclo(2β-5β)}; Renzetti et al., 1998) was synthesized by SibTech Inc. (Newington, CT, USA). NKA was obtained by EspiKem (Florence, Italy). All salts used were purchased from Merck and all other materials from Sigma. Nepadutant and MEN13510 were synthesized in Menarini Ricerche (Florence, Italy), and saredutant (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. CHO cell expression and culture

Site-directed mutagenesis of the NK_2 receptor cDNA was performed as previously described (Meini et al., 2004; Bellucci et al., 2004), and dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cell line CHO DUKX-B11 was used for stable transfection. Cells were cultured in α-modification essential Eagle's (αMEM) medium containing 10% dialysed foetal bovine serum (FBS) and 2 mM L-glutamine, and used for membrane preparations or inositol phosphates accumulation functional studies.

2.3. Membrane preparation and radioligand binding

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 cell pellet was suspended in Tris–HCl (50 mM), pH 7.4, containing bacitracin (0.1 mg/ml), chymostatin (0.01 mg/ml), leupeptin (5 μg/ml), and thiorphan (10 μM) (buffer A), and homogenized with a Polytron (PT 3000, Kinematica). 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 membrane protein concentration and frozen immediately in 1 ml aliquots by immersion in liquid nitrogen and there stored until use. The protein concentration was determined by the method of Bradford (1976).

Binding assay was performed at room temperature in a final volume of 0.5 ml, and with a different incubation time according to the used radioligand: 30 min for [^{125}I]NKA and [^{3}H]saredutant, and 60 min for [^{3}H]Nepadutant at the human tachykinin NK_2 receptor, 60 min for [^{3}H]SP and [^{125}I]NKA at the human tachykinin NK_1 receptor, and 90 min for [^{125}I][MePhe^7]NKB at the human NK_3 receptor (membranes of CHO expressing the hNK_3 receptor were provided by Amersham Biosciences, UK, and used according instructions). Each radioligand was used at a concentration less than the calculated K_d value ([^{125}I]NKA 0.2 nM, [^{3}H]saredutant 0.2 nM, [^{3}H]nepadutant 0.4 nM, [^{3}H]SP 0.1 nM, and [^{125}I][MePhe^7]NKB 0.16 nM) to give a bound less than 10% of the total added radioligand concentration, and 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 concentration in the assay was 1%, and it did not affect radioligands binding. Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard Instrument Company), pre-soaked for at least 2 h in polyethylenimine 0.3%, and using a MicroMate 96 Cell Harvester (Packard Instrument Company). The 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 (50 μl/well, Packard Instrument Company), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company).

2.4. Inositol phosphates determination

Cells were grown in 24-well tissue culture plates and labelled for 24 h with *myo*-[1,2-^{3}H] inositol (0.5 ml/well, 1 μCi/ml) in Iscove's modified Dulbecco's medium and Ham's F12 medium (1:1) containing 1% dialysed FBS and L-glutamine (2 mM). Different concentrations of NKA were incubated for 30 min at 37 °C in the stimulation buffer (PBS Ca/Mg free 135 mM, HEPES 20 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1.2 mM, EGTA 1 mM, glucose 11.1 mM, captopril 10 μM, BSA 0.05%) added with LiCl (25 mM) in the absence or in the presence of the antagonist, which was preincubated 15 min prior agonist administration. Total inositol phosphate levels were determined as previously described (Bellucci et al., 2004). Determinations were made in triplicate.

2.5. Data analysis

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

In functional experiments (inositol phosphates production) 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 4.0 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 equieffective concentrations of agonist in the presence and absence of antagonist (Kenakin, 1997).

Binding data were fitted by nonlinear regression using GraphPad Prism 4.0 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_d values were calculated for each used radioligand as IC_{50} – [radioligand]. Preliminary experiments were performed to select a radioligand concentration which had to be lower (from 2- to 10-fold) than the calculated IC50. K_i values were calculated from IC_{50} using the Cheng–Prusoff equation ($K_i = IC_{50}/(1+[\text{radioligand}]/K_d)$ according to the concentration and K_d of the used radioligand at each mutant receptor. Statistically significant differences in terms of ligand affinity values (K_i or K_d) at the mutant receptors were postulated on the basis of non overlapping 95% C.I. and F_{mut} index greater than 3.

3. Results

3.1. MEN13510 functional antagonist profile and selectivity for the human tachykinin NK_2 receptor

In CHO cells expressing the human tachykinin NK_2 receptor NKA (0.1 nM–1 μM) produced a concentration-dependent inositol phosphates accumulation with nanomolar potency, the EC_{50} value being 5.7 nM (4.4–7.5, 95% C.I.). MEN13510 did not affect the basal inositol phosphates accumulation up to 10 μM concentration. When MEN13510 was incubated 15 min before the agonist administration, it was able to shift the concentration–response curves to NKA in a concentration-dependent manner, leaving unaffected the produced maximal effect. Schild plot analysis was consistent with a competitive antagonism, the slope being −0.94 (−0.74–1.12, 95% C.I.) (Fig. 2). The antagonist potency of MEN13510 calculated as pK_B value was 7.8 ± 0.1.

MEN13510 binding inhibition curves were compared at the human tachykinin NK_1, NK_2, and NK_3 receptors by using the proper agonist radioligand: [^{3}H]SP, [^{125}I]NKA, and [^{125}I]MePhe^{7}NKB, respectively. MEN13510 inhibited the [^{125}I]NKA binding at the hNK_2 receptor similarly to the natural peptide ligand NKA (Fig. 3B) and the calculated K_i value was 6.4 nM (5.5–7.5, 95% C.I.). On the contrary, MEN13510 exhibited a low affinity for the other tachykinin receptor subtypes. MEN13510 inhibited with μM affinity both [^{3}H]SP binding (IC_{50} 1.06 μM, 0.6–1.8 95% C.I., Fig. 3A), and [^{125}I]NKA binding (IC_{50} 0.6 μM, 0.4–1.1, 95% C.I., data not shown) at the human tachykinin NK_1 receptor, and at 10 μM it inhibited by 31 ± 5% the [^{125}I]MePhe^{7}NKB binding at the human tachykinin NK_3 receptor.

3.2. Analysis of MEN13510 affinity at mutant human tachykinin NK_2 receptors

A number of 13 mutant receptors was investigated, all mutations 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

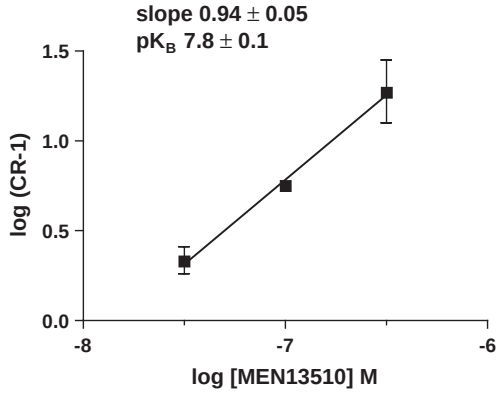

Fig. 2. MEN13510 antagonism on NKA induced inositol phosphates accumulation in CHO cells expressing the human tachykinin NK_2 receptor. Schild plot of MEN13510 antagonism. In abscissa the log of the antagonist molar concentration; in ordinate the log of the concentration ratio − 1 (CR − 1) of the agonist, where CR is the ratio of EC_{50} of NKA in the presence and absence of the indicated concentration of antagonist (see Data Analysis), the slope of Schild plot and the apparent affinity (pK_B) are indicated.

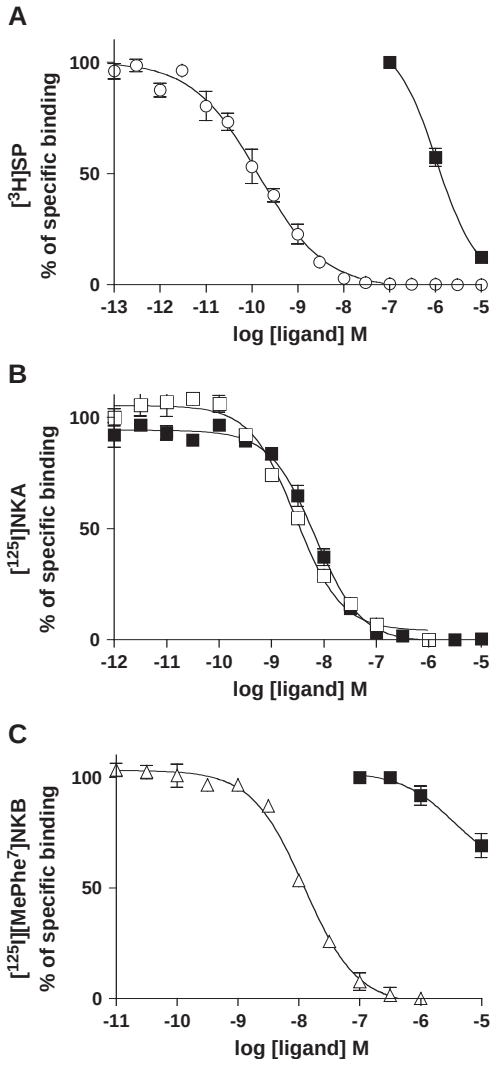

Fig. 3. Binding affinity of MEN13510 at the human tachykinin receptors. Heterologous competition binding curves for the antagonist ligand MEN13510 (closed squares) were performed in membrane preparations from stably transfected CHO cells expressing the human tachykinin NK_1, NK_2, or NK_3 receptor, using [^{3}H]SP, [^{125}I]NKA, and [^{125}I]MePhe^{7}NKB as radioligands, respectively. Homologous competition curves with unlabelled ligands were performed as control at the human tachykinin NK_1, NK_2, or NK_3 receptor with Substance P (open circles), NKA (open squares), and [MePhe^7]NKB (open triangles), respectively. Experimental conditions are described in the Material and Methods.

residues in the TM portion are schematically represented in Fig. 4. The affinity of MEN13510 at the wild type tachykinin NK_2 receptor was evaluated in inhibiting the binding of [^{125}I]NKA, [^{3}H]nepadutant, and [^{3}H]saredutant, and the calculated K_i values were 6.4, 12.3, and 26.3 nM, respectively (Table 1). No differences were observed in Hill coefficient values of displacement curves, thus indicating that ligands could bind according to a one-site binding model both at the wild type and the investigated mutant receptors.

MEN13510 binding inhibition curves at the Q109A, F112A, T171A, Y206A, W263A, and Y266F mutant tachykinin NK_2 receptors were comparable to those obtained at the wild type, thus ruling out a participation of these mutated residues to its receptor binding site.

Cys167 residue in the human tachykinin NK_2 receptor sequence corresponds to a Gly residue in the human tachykinin NK_1 receptor sequence (Takahashi et al., 1992). MEN13510 affinity at the Cys167Gly (C167G, TM4) mutant was measured at the [^{3}H]saredutant binding site where it was increased by ten-fold (K_i 2.1 nM, Table 1).

Ile202Phe (I202F) and C281Y are mutations which spontaneously occur in the rat and hamster tachykinin NK_2 receptor sequence (Sasai and Nakanishi, 1989): no differences were observed at these mutant receptors with all the tested radioligands. On the contrary, MEN13510 affinity at [^{125}I]NKA binding was decreased by 3-fold at the C281Y mutant, and by 9–13-fold at the I202F, whichever was the used radioligand.

F270A mutation has been previously described and assessed in our assay to impair the binding of both NKA and nepadutant radioligands, besides to reduce the affinity of the nonpeptide antagonist saredutant roughly by 5-fold (Renzetti et al., 1999). The calculated affinity of MEN13510 in inhibiting the [^{3}H]saredutant binding was reduced by 7-fold at the F270A mutant as compared with the wild type human tachykinin NK_2 receptor.

The affinity of MEN13510 was evaluated at the only mutated residues, located in the TM7, which crucially abolished the nonpeptide antagonist [^{3}H]saredutant binding (Huang et al., 1995; Renzetti et al., 1999). Tyr289Phe (Y289F) and Tyr289Thr (Y289T) mutations produced different effects on MEN13510, its affinity not being affected by the Y289F mutation at both [^{125}I]NKA and [^{3}H]nepadutant binding site. On the contrary, the Y289T and F293A mutations impaired by 32- and 13-fold, respectively, the MEN13510 affinity measured at the [^{3}H]nepadutant binding site. The binding behaviour of saredutant was compared to that of nepadutant and MEN13510 at these TM7 mutants. Heterologous inhibition curves performed with saredutant indicated a comparable reduction in affinity of 900- or 820-fold at the Y289F and Y289T mutants (K_i 387 nM, 235–638, 95% C.I., and K_i 353 nM, 237–526, 95% C.I., respectively), in agreement with the lack of detectable binding when the radioligand [^{3}H]saredutant was used (Table 1). A significant

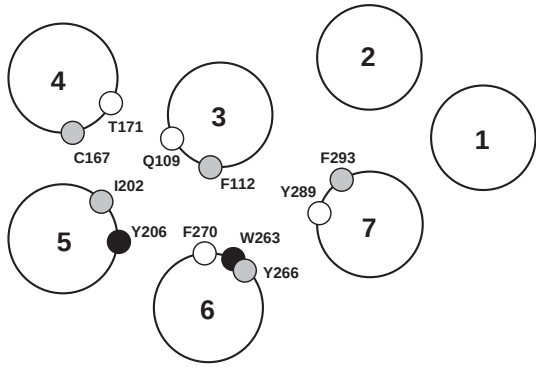

Fig. 4. Cross-sectional schematic sight of the seven transmembrane domains of the human tachykinin NK_2 receptor. The transmembrane domains (large circles 1 to 7) bear the investigated aminoacid residues (small circles). Aminoacid residues which lie at the same level in the helix are filled with the same colour, the white ones being in the outer portion and the darkest being deeper in the membrane. I202F, F270A, F293A, and Y289T are the mutations which determine a loss of affinity for the nonpeptide antagonist ligand MEN13510, whereas the mutation C167G determines an increase of MEN13510 affinity.

Table 1
Binding affinity at wild type and mutant human tachykinin NK_2 receptors

	[^{125}I]NKA			[^{3}H]Nepadutant			[^{3}H]Saredutant		
	NKA	MEN13510		Nepadutant	MEN13510		Saredutant	MEN13510	
	K_d [nM]	K_i [nM]	F_{mut}	K_d [nM]	K_i [nM]	F_{mut}	K_d [nM]	K_i [nM]	F_{mut}
Wild type	4.6 (3.2–6.2)	6.4 (5.5–7.5)	1	2.1 (1.6–2.8)	12.3 (8.1–18.8)	1	0.8 (0.6–0.9)	26.3 (18.3–37.7)	1
Q109A	6.3 (5.5–7.3)	8.7 (7.7–9.9)	1	2 (1.5–2.3)	13.3 (11.0–16.1)	1.1	1.4 (1.2–1.7)	14.6 (13.0–16.4)	0.6
F112A	n.t.	n.t.		23 (15.5–34)	17.3 (8.4–35.8)	1.4	n.t.	n.t.	
C167G	n.d.b.			n.d.b.			0.9 (0.1–3.0)	2.1 (1.2–3.7)	0.1
T171A	3.4 (2.2–4.3)	6.4 (5.5–7.4)	1	n.d.b.			0.8 (0.6–0.9)	43.7 (35.1–54.3)	1.7
I202F	3.8 (2.1–6.4)	61.3 (47.1–79.8)	10	0.7 (0.4–0.9)	160 (132–195)	13	1 (0.8–1.2)	244 (200–297)	9
Y206A	n.d.b.			n.d.b.			3.5 (2.5–5.7)	25.2 (12.3–51.6)	1
W263A	n.d.b.			n.d.b.			3.1 (1.7–4.1)	25 (8–64)	1
Y266F	4.4 (3.6–6.3)	9.41 (8.4–10.5)	1.5	n.d.b.			1.5 (1.2–1.8)	24 (18–32)	1
F270A	n.d.b.			n.d.b.			4.5 (2.1–6.8)	177 (138–226)	7
C281Y	5.5 (3.5–7.5)	19 (13–30)	3	2.3 (2.0–2.8)	n.t.		0.7 (0.5–0.9)	n.t.	
Y289F	4 (3.1–4.8)	3.5 (3.12–4.04)	0.6	7.7 (6.3–11.2)	16.5 (12.8–21.2)	1.3	n.d.b.		
Y289T	n.d.b.			30 (22–38)	393 (328–470)	32	n.d.b.		
F293A	n.t.	n.t.		7.2 (5.5–9.2)	80 (68.2–93.7)	13	n.t.	n.t.	

Experiments were carried out on membrane preparations from pooled clones of CHO cells stably expressing the wild type or mutant human tackykinin NK_2 receptors, by using the peptide agonist [^{125}I]NKA, the cyclic peptide antagonist [^{3}H]nepadutant, and the nonpeptide antagonist [^{3}H]saredutant as radioligands, as described under the Materials and Methods. Radioligands K_d constant affinity values were calculated by means of homologous inhibition experiment and expressed in nM concentration, for each receptor. K_i values of the competing ligand MEN13510 (in nM concentration) were calculated from heterologous inhibition experiments according to the concentration and K_d of the used radioligand for each mutant receptor (see Materials and Methods). F_{mut} is calculated as K_i(mutant receptor)/K_i(wild type human tachykinin NK_2 receptor), and corresponds to fold decrease in MEN13510 affinity. Values are mean and 95% confidence intervals in parenthesis of 3 experiments, each one performed in duplicate. n.d.b., not detectable binding. n.t., not tested.

reduction in saredutant affinity (24-fold, K_i 10.3 nM, 8.8–12.1, 95% C.I.) was observed also at the F293A mutant.

3.3. Analysis of MEN13510 affinity at the human tachykinin NK_2 receptor β isoform

A splice variant of the human tachykinin NK_2 receptor has been recently identified (Candenas et al., 2002), namely β isoform. MEN13510 was evaluated in inhibiting the binding of [^{3}H]saredutant, already indicated as the only radioligand able to bind to the human tachykinin NK_2 receptor β isoform (Bellucci et al., 2004). MEN13510 did inhibit in a concentration-dependent manner the [^{3}H]saredutant binding in cells membranes expressing the human tachykinin NK_2 receptor β isoform (Fig. 5), and the curve was fitted by a sigmoidal nonlinear regression (slope 0.62, 0.466–0.76 95% C.I.) with IC_{50} value 2.2 nM (1.4–3.5, 95% C.I.), and the calculated K_i was 1.99 nM (1.26–3.16, 95% C.I.). These data best fitted according with a two-binding sites model (R squared 0.9868), and the resulting IC_{50} values were 0.15 nM (0.04–0.057, 95% C.I.) and 7.8 nM (3.9–15.3, 95% C.I.).

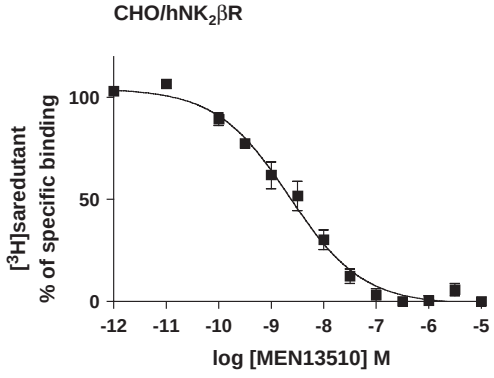

Fig. 5. Binding affinity of MEN13510 for the human tachykinin NK_2 receptor β isoform. Heterologous competition binding curves for the antagonist ligand MEN13510 (closed squares) were performed in membrane preparations from stably transfected CHO cells expressing the human tachykinin NK_2 receptor β isoform, using [^{3}H]saredutant (0.4 nM) as radioligand.

4. Discussion

The present study shows that MEN13510 is an antagonist ligand endowed with nanomolar affinity for the human tachykinin NK_2 receptor, whereas it possesses low affinity for the other human tachykinin receptor subtypes. Since the human tachykinin NK_1 receptor can be bound with high affinity from both SP and NKA radioligands, which can select different receptor conformers (Hastrup and Schwartz, 1996), the present findings indicate that MEN13510 possesses micromolar affinity for the human tachykinin NK_1 receptor, independently from the unique agonist selected receptor conformation. Present results indicate MEN13510 as a competitive antagonist in inhibiting the NKA induced inositol phosphates accumulation (pK_B 7.8) in cells expressing the human tachykinin NK_2 receptor, being 4-fold less potent than the cyclic peptide antagonist nepadutant (pA_2 8.4 at 100 nM concentration, data not shown), and 100-fold less potent then the nonpeptide antagonist saredutant

(pA_2 10 at 10 nM concentration, data not shown). Moreover, at least in this bioassay, per se MEN13510 did not show negative efficacy up to micromolar concentration. It must be considered that inverse agonism cannot be ruled out, since it can be detected only in bioassays endowed with high receptor constitutive activity (Kenakin, 2004).

In the G protein-coupled receptors, the ligand binding crevice is usually located in the hydrophobic core between the extracellular loops and the outer portions of TMs 3, 4, 5, 6, and 7, and this appears to be the case also for the human tachykinin NK_2 receptor (Almeida et al., 2004, for review). In the present study we describe the affinity of MEN13510 at point mutated human tachykinin NK_2 receptors which have been previously shown to impair the binding of other antagonist ligands, such as the bicyclic peptide nepadutant (Catalioto et al., 1998), and the monocyclic peptide MEN11558 (Renzetti et al., 1999; Giolitti et al., 2000, 2002; Giannotti et al., 2000), from which structures stems MEN13510 (see Introduction).

None of the studied mutated receptor residues resulted crucial for this newly presented antagonist ligand, since the greater reduction in affinity was 32-fold (Y289T), but we advance that other discriminants may be more fundamental for MEN13510 binding to the human tachykinin NK_2 receptor.

Critical residues for receptor interaction with the cyclic peptidic ligands nepadutant and MEN11558 were described to be overall superimposable, at level of TMs 4, 6, and 7 (Giolitti et al., 2000, 2002). In particular a residue critical for binding of nepadutant was the T171 located in the TM4 (Fig. 4), as the Ala substitution abolished its binding (Giolitti et al., 2000). On the contrary, when nepadutant was compared to the monocyclic MEN11558 compound in inhibiting the NKA binding site at the T171A mutant, their affinity was comparable to that measured at the wild type (Giolitti et al., 2002), as detected for MEN13510 in the present study. For this reason no conclusions can be drawn about the direct participation of this residue in MEN13510 interaction with the human tachykinin NK_2 receptor. A further mutation which drastically impaired the affinity of both cyclic peptides was the C167G ($\geq$700-fold, Giolitti et al., 2002), which lies roughly one turn down the T171 residue. MEN13510 affinity at this mutant was indeed increased (10-fold), suggesting that C167 residue could represent a steric hindrance for the interaction with the receptor by this ligand.

We already presented data on some pseudopeptide antagonist ligands which possess low affinity for the rat tachykinin NK_2 receptor (Meini et al., 2004), and have an affinity impairment at the I202F mutation of the human tachykinin NK_2 receptor (a Phe residue does occur in the TM5 of rat tachykinin NK_2 receptor sequence). The drop in affinity described for MEN13510 at this mutant (10-fold), as well as at the C281Y (3-fold) (residue located in the extracellular loop between TM6 and TM7, substituted with the corresponding aminoacid reported for the rat tachykinin NK_2 receptor) allows us to predict a species selectivity for this compound.

Further differences were detected amongst mutations in the sequence of TM3 (F112A) and TM6 (W263A, Y266F, F270A) which in general were shown to impair, at various extents, the binding of the cyclic peptide antagonists nepadutant and MEN11558 (Giolitti et al., 2000, 2002). Again present findings indicate that MEN13510 binding differs from that of cyclic peptide antagonists. In fact, amongst the mutated residues belonging to TM3 and TM6, which face each other, only the F270A mutation does hinder somehow the MEN13510 binding (7-fold). Altogether these data indicate that the binding site of MEN13510 is not buried into the same hydrophobic pocket relevant for the peptidic ligands, or alternatively that other receptor discriminants are crucial for the high affinity interaction of this ligand.

It was previously shown that mutations of the Y289 residue were detrimental for the nonpeptide antagonist saredutant (Huang et al., 1995) whichever was the aminoacidic substitution (Giolitti et al., 2000), as confirmed by present findings by means of radioligand and heterologous inhibition binding experiments. On the other hand, the only aromatic moiety of Y289 appeared to be important for the binding of NKA (Renzetti et al., 1999), besides other nonpeptide (GR159897, Huang et al., 1995) or pseudopeptide antagonists (MEN13918, Meini et al., 2004). Similarly to NKA, MEN13510 receptor interaction seems to be altered when the Y289 residue is substituted with a Thr residue (Y289T, 30-fold decrease in affinity), but not a Phe residue (Y289F). These data suggest a lipophilic interaction between the receptor and the ligand, and a non participation of the hydroxylic moiety of Y289. One turn down the Y289 lies the F293 residue, which mutated to alanine impaired the affinity of both MEN13510 (10-fold) and saredutant (24-fold). Taken together these observations indicate a pool of aromatic residues in TMs 6 and 7 (F270, Y289, and F293), which have been described to participate in the binding site of NKA, which play a role in the hydrophobic MEN13510–receptor interaction.

As a whole the results presented in this study indicate that the binding site of MEN13510 is, at least in part, overlapping to that described for NKA or saredutant (Almeida et al., 2004, for review). Even if we have found binding differences between the nonpeptide antagonist saredutant and MEN13510 within the series of mutant receptors we considered, the most relevant differences were found between peptide and nonpeptide ligands. It seems therefore that new nonpeptidic structures could be found as tachykinin NK_2 receptor ligands, without deriving them from the overused lead saredutant, as for other tachykinin NK_2 receptor antagonists (YM-35375, Kubota et al., 1998; UK224671, compound 33 in MacKenzie et al., 2002).

It has recently been discovered a splice variant of the human tachykinin NK_2 receptor, namely β isoform, lacking of the second intracellular loop, the TM4, and the third extracellular loop, and widely coexpressed (in terms of mRNA) with the wild type (referred to α isoform) (Candenas et al., 2002). On the other hand, we have recently shown that this receptor β isoform is poorly expressed on cell membrane surface, and is not able to bind the agonist NKA or the antagonist nepadutant radioligands, whereas it specifically binds the nonpeptide ligand saredutant (Bellucci et al., 2004). In agreement with the lack of interaction by MEN13510 with the studied receptor residues located in the TM4, and its similarity with the saredutant binding site at the wild type receptor α isoform, this compound retains a nanomolar affinity for the β isoform of the human tachykinin NK_2 receptor, whatever the physiological meaning can be.

In conclusion, the present study extensively investigates the pharmacology of MEN13510 as a high affinity selective antagonist ligand for the human tachykinin NK_2 receptor, and since it retains nanomolar affinity for both receptor isoforms, can be an interesting tool for studying the tachykinin NK_2 receptor β isoform.

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