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MEN16132, a novel potent and selective nonpeptide antagonist for the human bradykinin B_2 receptor. In vitro pharmacology and molecular characterization

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Abstract

The pharmacological characterization of the novel nonpeptide antagonist for the B_2 receptor, namely MEN16132 (4-(*S*)-Amino-5-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2*H*-4-pyranylcarbonyl}piperazino)-5-oxopentyl](trimethyl)ammonium chloride hydrochloride) is presented.

The affinity of MEN16132 for the bradykinin B_2 receptor has been investigated by means of competition studies at [^{3}H]bradykinin binding to membranes prepared from Chinese Hamster Ovary (CHO) cells expressing the human bradykinin B_2 receptor (pK_i 10.5), human lung fibroblasts (pK_i 10.5), guinea pig airways (pK_i 10.0), guinea pig ileum longitudinal smooth muscle (pK_i 10.2), or guinea pig cultured colonic myocytes (pK_i 10.3). In all assays MEN16132 was as potent as the peptide antagonist Icatibant, and from 3- to 100-fold more potent than the reference nonpeptide antagonists FR173657 or LF16-0687. The selectivity for the bradykinin B_2 receptor was checked at the human bradykinin B_1 receptor ($pK_i < 5$), and at a panel of 26 different receptors and channels.

The antagonist potency was measured in functional assays, i.e., in blocking the bradykinin induced inositolphosphates (IP) accumulation at the human (CHO: pK_B 10.3) and guinea pig (colonic myocytes: pK_B 10.3) B_2 receptor, or in antagonizing the bradykinin induced contractile responses in human (detrusor smooth muscle: pK_B 9.9) and guinea pig (ileum longitudinal smooth muscle: pK_B 10.1) tissues. In both functional assay types MEN16132 exerted a different antagonist pattern, i.e., surmountable at the human and insurmountable at the guinea pig bradykinin B_2 receptors.

Moreover, the receptor determinants important for the high affinity interaction of MEN16132 with the human bradykinin B_2 receptor were investigated by means of radioligand binding studies performed at 24 point-mutated receptors. The results obtained revealed that residues in transmembrane segment 2 (W86A), 3 (I110A), 6 (W256A), and 7 (Y295A, Y295F but not much Y295W), were crucial for the high affinity of MEN16132. In conclusion, MEN16132 is a new, potent, and selective nonpeptide bradykinin B_2 receptor antagonist.

Keywords: Binding site; Cyclic peptide; G protein-coupled receptor; Icatibant; Site-directed mutagenesis

1. Introduction

Kinins, such as bradykinin (H–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH) and kallidin (Lys-bradykinin), are important peptidic mediators of pain, inflammation, and

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cardiovascular homeostasis, being released from kininogen by activation of both plasma and tissue kallikreins (Bhoola et al., 1992). In a variety of cell types the effects of bradykinin or Lys-bradykinin are mediated by the B_2 receptor, belonging to the superfamily of G protein coupled receptors (Hess et al., 1992). On the other hand, bradykinin or Lys-bradykinin metabolites lacking the C-terminal Arg, act on the bradykinin B_1 receptor subtype, which is expressed under chronic inflammatory conditions (Marceau, 1995).

Because of the bradykinin B_2 receptor role in mediating pain and inflammation, potent and selective nonpeptide antagonists have been identified in recent years (Altamura et al., 1999) in order to achieve a greater drug bioavailability in comparison with peptidic compounds.

In this study we present the pharmacological outline of a novel nonpeptide antagonist for the bradykinin B_2 receptor, namely MEN16132 (Fig. 1) (4-(*S*)-Amino-5-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2*H*-4-pyranylcarbonyl}piperazino)-5-oxopentyl](trimethyl)ammonium chloride hydrochloride), which has structural similarities with the previously presented antagonists FR173657 (Asano et al., 1997) and LF16-0687 (Pruneau et al., 1999). Whereas structure activity relationships studies which led to the discovery of MEN16132 will be presented elsewhere (Giolitti et al., manuscript in preparation), in the current study we have extensively characterized: 1) the selectivity for the human bradykinin B_2 receptor, as compared with the human bradykinin B_1, and to other G-protein-coupled receptors and ion channels; 2) the affinity of this ligand for the guinea pig bradykinin B_2 receptor, and 3) the antagonist profile of MEN16132 in different functional assays (IP accumulation and contractility responses) both at the human and guinea pig bradykinin B_2 receptors. Moreover, the structural receptor determinants important for the high affinity interaction of MEN16132 with the human bradykinin B_2 receptor have been investigated by means of radioligand binding studies performed at 24 mutant receptors, previously used for the characterization of both antagonist and agonist bradykinin B_2 receptor ligands (Marie et al., 2001; Meini et al., 2002, 2004; Cucchi et al., 2002; Bellucci et al., 2003).

Throughout the study the results obtained with MEN16132 have been compared with those obtained with other reference peptidic (Icatibant and MEN11270, Hock et al., 1991; Meini et al., 1999) and non-peptidic antagonists (FR173657 and LF16-0687, Asano et al., 1997; Pruneau et al., 1999).

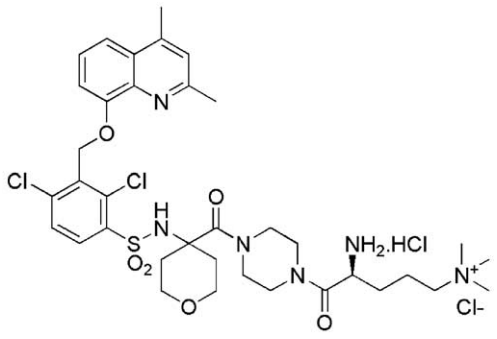

Fig. 1. Structure of MEN16132 (4-(*S*)-Amino-5-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2*H*-4-pyranylcarbonyl}piperazino)-5-oxopentyl](trimethyl)ammonium chloride hydrochloride).

2. Materials and methods

2.1. Materials

[^{3}H]bradykinin (specific activity 90 Ci/mmol), [^{3}H][desArg^9]Lys-bradykinin (specific activity 80 Ci/mmol), and *myo*-[1,2-^{3}H] inositol (specific activity 60–80 Ci/mmol) were provided by Perkin Elmer New England Nuclear, and [^{3}H]MEN11270 (specific activity 30 Ci/mmol) (H–DArg–Arg–Pro–Hyp–Gly–Thi–c(Dab–DTicOicArg)c(7γ–10α); Meini et al., 1999) was synthesized by SibTech Inc. (Newington, CT, USA). Bradykinin and [desArg^9]Lys-bradykinin were obtained from Neosystem (Strasbourg, France), and bestatin from Peninsula (Peninsula Laboratories Europe, Cheshire, UK). Thiorphan was from Bachem (Essex, UK), captopril from Sigma, (Dorset, UK). All salts used were purchased from Merck (Darmstadt, Germany). MEN16132 and reference antagonists were synthesized in Menarini Ricerche (Chemistry Departments of Florence and Pomezia, Italy), dissolved in dimethylsulphoxide up to 1 mM and stored at −25 °C.

2.2. Isolation and culture of colonic myocytes

All procedures and protocols were approved by the Local Ethical Committee. Male Dunkin–Hartley guinea pigs (Charles River) weighing 250–400 g were used. A 3-cm long piece of proximal colon from one animal was excised and placed in warmed (37 °C) and oxygenated (95% O_2, 5% 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). Mucosa-free circular muscle strips (0.5–0.8 mm wide and 10–15 mm long) were excised and washed five times in culture medium (Minimum essential Eagle's medium α modification, α-MEM) containing penicillin (100U/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml) (wash medium). Then tissue pieces were digested by incubating them at 37 °C in 10 ml of wash medium added with collagenase type II (0.15% wt/vol), pronase (0.015% wt/vol), soybean trypsin inhibitor type II (0.01% wt/vol), bovin serum albumin (BSA, 0.1% wt/vol), and gentamicin (4 μg/ml). After 20 min of digestion the supernatant was transferred through filtration on Nytex (BDH, nylon mesh 250 μm) in 5 ml of fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and the remaining tissue was digested twice as above. Cells suspensions were then pooled and centrifuged (5 min, 300 g) at room temperature. The pelletted cells were suspended in culture medium (αMEM medium containing penicillin, streptomycin, fungizone at the above concentrations, and non essential aminoacids (1%), ECGS (30 μg/ml), and FBS 2%), and plated onto 4 culture flask (75 cm^2). The culture medium was changed every 2 days and in these conditions cells reached the confluence after 7 days, than cells were seeded in 24-wells

(400,000 cells/ml for IP assay), or in 175 cm^2 flasks (for membrane preparation).

2.3. Cells membrane preparation

CHO cells were stably transfected as previously described (Meini et al., 2004). All cells were cultured in Iscove's modified Dulbecco's Medium (IMDM) with 2 mM L-glutamine and FBS (10%). Cells at confluence (transfected CHO cells, human lung fibroblasts, HLF-1, or guinea pig colonic myocytes) were harvested by incubating at 37 °C with *N*-[2-hydroxy-ethyl] piperazine-N′-[2-ethanesulphonic acid] (10 mM), ethylenediaminetetraacetate 1 mM, in Hanks Buffered Salt Solution (pH 7.4) containing a cocktail of peptidase inhibitors: 1,10 phenanthroline (1 mM), ethylene glycol *bis* (β-aminoethyl ether)-N, N, N′,N′-tetraacetic acid (1 mM), captopril, leupeptin, soy bean trypsin inhibitor, DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (1 μM each), chymostatin (3.3 μM), phenylmethyl–sulphonyl fluoride (0.1 mM), and bacitracin (140 μg/ml). Cells were then washed in *N*-tris[hydroxymethyl] methyl-2-aminoethanesulphonic acid (10 mM, pH 7.4, at 4 °C), containing the above described peptidase inhibitors cocktail, and homogenized with a Polytron (PT 3000, Kinematica) for 30 s on ice. Homogenate was centrifuged at 45,000 *g* for 45 min (4 °C). The pellet was resuspended to obtain 7.5 mg/ml 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) using a Bio-Rad kit. Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

2.4. Tissue membrane preparation

Fresh tissues (guinea pig airways, i.e., lung and bronchi, or guinea pig ileum longitudinal smooth muscle) were placed in *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES, 10 mM, pH 7.4, at 4 °C) additioned with a cocktail of peptidase inhibitors: 1,10 phenanthroline (1 mM), ethylene glycol *bis* (β-aminoethyl ether)-N, N, N′,N′-tetraacetic acid (1 mM), captopril, leupeptin, soy bean trypsin inhibitor, DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (1 μM each), chymostatin (3.3 μM), phenylmethyl–sulphonyl fluoride (0.1 mM), and bacitracin (140 μg/ml). The tissues were minced and homogenized with a Polytron (PT 3000, Kinematica), set at 15,000 r.p.m. for 30 s in 10 ml/g of the above buffer. The homogenate was centrifuged at 2500 *g* for 10 min to remove cellular debris. The supernatant was homogenized and centrifuged at 45,000 *g* (4 °C) for 30 min. The pellet was resuspended in binding buffer and frozen immediately in 2 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).

Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

2.5. Radioligand binding experiments

The buffer used for all binding experiments was *N*-tris [hydroxymethyl]methyl-2-aminoethanesulphonic acid (10 mM, pH 7.4) containing 1,10-phenanthroline (1 mM), bacitracin (140 μg/ml), and bovine serum albumin (1 g/l). Binding assays were performed at room temperature in a final volume of 0.5 ml, and an incubation time of 60 min was used. The radioligands concentration employed was comparable with their calculated K_d value (0.1–0.2 nM). At this concentration the bound was less than 10% of the total added radioligand concentration, and the specific binding represented approximately 70–80% of the radioligand total binding. Competing ligands were tested in a wide range of concentrations (1 pM–10 μM). Non-specific binding was defined as the amount of labelled ligand bound in the presence of 1 μM of the appropriate unlabelled ligand. Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates GF/B (Packard Instrument Company), presoaked for at least 2 h in polyethylenimine 0.6%, 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 (50mM, 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).

The binding affinity of MEN16132 for a range of 26 different receptors and for Na^+, Ca^{2+}, K^+ and Cl^-channels (see Results) was measured according to methods established by Cerep (Le Bois L'Evêque BP 1, 86600 Celle l'Evescault, France).

2.6. Inositol phosphates (IP) determination

Cells (CHO expressing the human B_2 receptor or guinea pig colonic myocytes) were grown in 24-well tissue culture plates and labeled for 24 h with *myo*-[1,2-^{3}H] inositol (0.5 ml, 1 μCi/ml) in IMDM and Ham's F12 Medium (F12) (1 : 1) containing FBS (1%) and L-glutamine (2 mM). Different concentrations of agonists were incubated for 30 min at 37 °C in the stimulation buffer (phosphate buffered saline 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). Total inositol phosphate (IP) levels were determined as previously described (Meini et al., 2004). Determinations were made in triplicate.

2.7. Smooth muscle contractility assays

All experiments were performed under authorization of the University of Ferrara Ethical Committee.

Human detrusor muscle strips were excised from the dome of the urinary bladder of 11 patients with a mean age of 59 (53–68) years, undergoing cystectomy because of a carcinoma of the bladder base. No patient received radio-or chemotherapy before intervention. In all patients, preanesthetic

medication was intramuscular atropine (1 mg) and diazepam (10 mg). Anesthesia was induced by sodium thiopental (500 mg i.v.) and maintained with N_2O/O_2 (1 : 2) and halothane (0.6–1%). The patients received pancuronium bromide (6 mg i.v.) during induction of anesthesia. All specimens appeared macroscopically normal without signs of tumor or inflammation. The tissues were placed in ice-cold Krebs'solution (mM composition, see above) within 2–3 min after surgical removal.

Guinea pig ileum longitudinal smooth muscle-myenteric plexus smooth muscle preparation was prepared as previously described (Meini et al., 2000b).

Smooth muscle strips were placed in organ baths (5 ml capacity) containing oxygenated and gassed (95% O_2, 5% CO_2) Krebs' solution. Mechanical activity was isotonically recorded (load: 5 mN guinea pig ileum, 10 mN human detrusor; Basile 7050 pen recorder). After 1 h equilibration period, bradykinin (1 μM, guinea pig ileum) or KCl (80 mM, human detrusor) was administered 3–4 times to preparations, every 20 min interval, to test sensibility and reproducibility of the contractile response. Afterwards, a cumulative concentration–response curve to bradykinin (1 nM–1 μM) was constructed. At the end of each curve, the maximal contractile response of the preparation was evaluated by administration of KCl (80 mM). After washout and recovery of basal tone, the concentration–response curve to bradykinin was repeated in the presence of the receptor antagonist. Peptidase inhibitors (thiorphan, bestatin, and captopril, 1 μM) were added 15 min prior to determination of the bradykinin induced concentration–response curve, and antagonists contact time was 15 min.

The reversibility of B_2 receptor blockade produced by MEN16132 was evaluated in the guinea pig ileum preparation as follows: bradykinin (100 nM) was administered to the preparations at 30 min intervals, until reproducible contractile responses were obtained (generally 3 administrations). At this time MEN16132 (1 nM) or vehicle were 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.

2.8. Analysis of data

Each value in the text is mean ± S.E.M or the mean and 95% confidence intervals (C.I.) in parenthesis.

Binding data were fitted by nonlinear regression using GraphPad Prism 4.0 (GraphPad, San Diego, CA), in order to determine i) the maximum binding site density (B_{max}) and the equilibrium dissociation constant (K_d) from saturation experiments, and ii) 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 ($K_i = IC_{50}/(1 + [\text{radioligand}]/K_d)$) according to the concentration and K_d of the used radioligand in each receptor system (cell, tissue, or mutant). Statistically significant differences in terms of ligand affinity values at the mutant receptors were postulated on the basis of non overlapping 95% C.I. and F_{mut} index greater than 3.

Functional data were fitted by sigmoidal nonlinear regression (GraphPad Prism 4.0) to determine the agonist concentration producing the 50% (EC_{50}) of the maximal response (E_{max}) from the concentration–response curves.

The nature of the MEN16132 interaction with the receptor was checked by the Schild regression as follows: antagonist-induced parallel shifts of concentration–response curves to the agonist were calculated as the ratio (concentration-ratio, CR) of equieffective concentrations of agonist (EC_{50}) obtained in the presence and in the absence of antagonist. Estimates of log [CR−1] were plotted against log [antagonist concentration] (Arunlakshana and Schild, 1959). Antagonism providing plots with linear regression lines and slopes not significantly different from unity was considered to act competitively. The affinity of competitive antagonism was expressed in terms of pK_B calculated from the equation: $pK_B = \log [CR-1] - \log [\text{antagonist concentration}]$ (Kenakin, 1997a).

When MEN16132 caused nonparallel rigthward shifts of the concentration–response curve to bradykinin, and decreased its E_{max}, the estimate of antagonist affinity was calculated by the method described for non-competitive and/or pseudoirreversible antagonists as described by Kenakin (1997b). 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 derived from the equation:

$$K_B = [B]/\text{slope} - 1. \quad (1)$$

In order to obtain more accurate estimates of K_B we selected the experiments in which the antagonist depressed the agonist control E_{max} more than 50%.

3. Results

3.1. Radioligand binding studies

3.1.1. Binding affinity and selectivity for the human B_2 receptor

MEN16132 concentration dependently inhibited the binding of [^{3}H]bradykinin to CHO cell membranes expressing the human bradykinin B_2 receptor (K_d 0.07 nM, B_{max} 330 fmol/mg of proteins), and the determined pK_i value was 10.5 ± 0.05 (n = 12, Hill slope 1.5, 1.3–1.7 95% C.I.). Overlapping data were obtained when inhibition binding experiments were performed with membranes of human lung fibroblasts (HLF-1) which natively espress the B_2 receptor ([^{3}H]bradykinin K_d 0.1 nM, B_{max} 190 fmol/mg of proteins, pK_i value of MEN16132 10.5, 10.4–10.6, 95% C.I.; Hill slope 1.6, 1.4–1.8, 95% C.I.) (Fig. 2, Table 1). On the contrary, MEN16132 affinity determined in inhibiting the agonist [^{3}H][desArg^9]Lys-bradykinin radioligand binding at the human bradykinin B_1 receptor was much lower (more than 5 orders of magnitude, Fig. 2), both at the recombinant (CHO: [^{3}H][desArg^9]Lys-bradykinin K_d 0.16 nM, B_{max} 64 fmol/mg of proteins) and native receptor

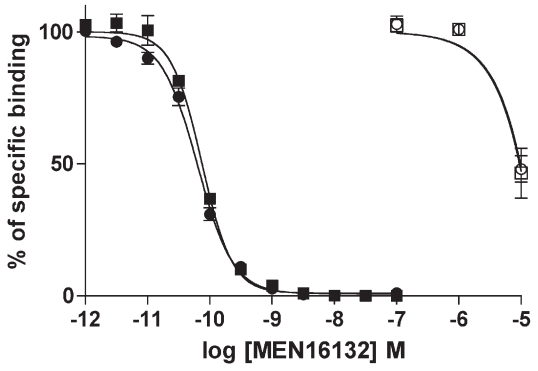

Fig. 2. Binding affinity of MEN16132 at the human bradykinin receptor Heterologous inhibition binding curves for the antagonist ligand MEN16132 were performed in membrane preparations from CHO cells expressing the human bradykinin B_2 or B_1 receptor (circles), or human lung fibroblasts (squares). [^{3}H]bradykinin or [^{3}H][desArg^9]Lys-bradykinin were used as selective radioligands for the B_2 (closed symbols) and B_1 receptor (open symbols), respectively. Experimental conditions are described in Material and methods. pK_i affinity values are reported in Table 1. Data points represent the mean±S.E.M. of 3 independent experiments, each one performed in duplicate.

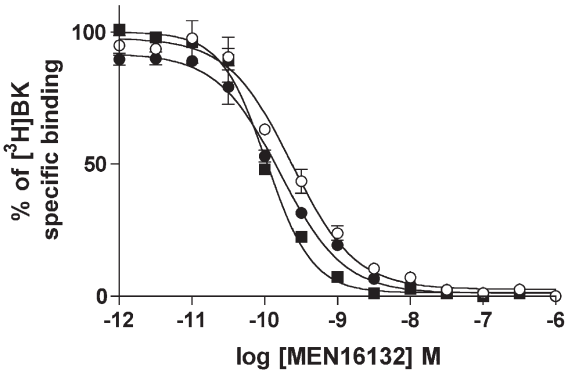

Fig. 3. Binding affinity of MEN16132 at the guinea pig bradykinin B_2 receptor Heterologous competition binding curves for the antagonist ligand MEN16132 were performed in membrane preparations from guinea pig airway (open circles), longitudinal ileum smooth muscle (closed circles), and colonic myocytes (closed squared). [^{3}H]bradykinin was used as radioligand according to the described experimental conditions, and pK_i affinity values are reported in Table 1. Data points represent the mean±S.E.M. of 3 independent experiments, each one performed in duplicate.

(HLF-1: [^{3}H][desArg^9]Lys-bradykinin K_d 0.02 nM, B_{max} 30 fmol/mg of proteins).

The affinity of MEN16132, as determined at the recombinant (CHO) and native (HLF-1) human bradykinin B_2 receptor, was compared to that obtained with other reference antagonists in the same set of experiments: the rank order of potency obtained was MEN16132 ≥ Icatibant > MEN11270 ≥ LF16-0687 > FR173657 (pK_i values in Table 1).

The affinity of MEN16132 was determined for many receptors and ion channels by using conventional radioligand binding techniques. At 1 μM concentration MEN16132 did not inhibit the radioligand binding measured at the human receptors adenosin A_1, adenosin A_{2A}, adrenergic α_1, adrenergic α_2, adrenergic β_1, angiotensin AT_1, calcitonin-gene-related peptide, interleukin-1β, tumor necrosis factor α, chemokine receptor type 1, chemokine receptor type 2, neurokinin 1, neurokinin 2, neurokinin 3, neuropeptide Y, thromboxane A_2, prostaglandin I_2, opioid-like receptor 1, serotonin, muscarinic, neurotensin, somatostatin, bombesin and vasoactive intestinal peptide receptors. MEN16132 had no measurable binding affinity (pIC_{50} < 5.5) for Ca^{2+}channels (L-type: dihydropiridine, verapamil, diltiazem sites; N-type), K^+channels (ATP-, voltage-, and Ca^{2+}-dependent), Na^+channel (site 2) or Cl^-(picrotoxin) channel.

3.1.2. Binding affinity for the guinea pig B_2 receptor

MEN16132 displayed high affinity in inhibiting [^{3}H] bradykinin binding both in guinea pig airway ([^{3}H]bradykinin K_d 0.13 nM), and ileum ([^{3}H]bradykinin K_d 0.13 nM) tissues, or colonic myocytes ([^{3}H]bradykinin K_d 0.07 nM) membranes, the pK_i values being 10.0 (9.9–10.1, 95% C.I.; Hill slope 0.9, 0.7–1.1 95% C.I.), 10.2 (10.1–10.25, 95% C.I.; Hill slope 0.9, 0.7–1.1 95% C.I.), and 10.3 (10.1–10.5, 95% C.I.; Hill slope 1.5, 0.9–1.9, 95% C.I.), respectively (Fig. 3, Table 1). The affinity determined for MEN16132 was compared to that obtained with other reference antagonists, and the rank order of potency was: MEN16132 = Icatibant > MEN11270 ≥ LF16-0687 > FR173657 (pK_i values in Table 1).

Table 1
Antagonists binding affinities at the human and guinea pig bradykinin B_2 receptor

	[^{3}H]bradykinin binding							
	Human bradykinin B_2 receptor				Guinea pig bradykinin B_2 receptor			
	CHO		HLF-1		Airways		Ileum	
	pK_i	(95% C.I.)	pK_i	(95% C.I.)	pK_i	(95% C.I.)	pK_i	(95% C.I.)
MEN16132	10.5	(10.4–10.6)	10.5	(10.4–10.6)	10	(9.9–10.1)	10.2	(10.1–10.3)
Icatibant	10.1	(9.9–10.2)	10.6	(10.4–10.8)	9.8	(9.5–10.1)	10.5	(10.3–10.7)
MEN11270	9.6	(9.5–9.7)	10.3	(10.2–10.4)	9.6	(8.9–10.3)	10.2	(9.9–10.5)
LF16-0687	9.7	(9.7–9.8)	9.8	(9.6–10)	9.5	(9.4–9.7)	9.7	(9.6–9.8)
FR173657	7.8	(7.6–7.9)	n.t.		8.3	(8.1–8.6)	8.7	(8.6–8.7)

Experiments were carried out on membrane preparations from CHO cells stably expressing the human bradykinin B_2 receptors (CHO/hB_2), human lung fibroblasts (HLF-1), guinea pig airway and ileum tissues, by using the peptide agonist [^{3}H]bradykinin as radioligand. K_i values of antagonist ligands were calculated from heterologous inhibition experiments according to the concentration and K_d of the radioligand. Data are from 3 experiments, each one performed in duplicate. n.t. not tested.

3.2. Functional studies

3.2.1. Antagonist potency at the human B_2 receptor

In CHO cells stably transfected with the human bradykinin B_2 receptor, bradykinin (0.01 – 100 nM) induced a concentration-dependent stimulation of IP formation, the agonist induced maximal increase over the basal output being 6.7±0.8 fold. MEN 16132 did not modify the basal IP accumulation up to μM concentration, and produced a concentration-dependent (1,10 and 100 nM) rightward shift of bradykinin response curve without significantly affecting the agonist induced maximal IP formation (Fig. 4A). The Schild plot analysis (slope of linear regression 1.1, 0.7–1.5, 95% C.I.) was indicative of a competitive antagonism, and the calculated affinity as pK_B value was 10.3 (9.9–10.7, 95% C.I.).

In the human detrusor smooth muscle preparation bradykinin (0.1 nM–10 μM) produced a concentration-dependent contraction, the maximal response averaging 76±13% of that induced by KCl (80 mM). MEN16132 induced a concentration-dependent (1–10–30 nM) rightward shift of the concentration–response curve to bradykinin leaving unaltered the E_{max} (Fig. 4B), and the calculated affinity as pK_B value was 9.9 (9.5–10.7, 95% C.I.). The Schild plot analysis was indicative of a competitive antagonism, the slope of linear regression being 0.94 (0.6–1.2, 95% C.I.).

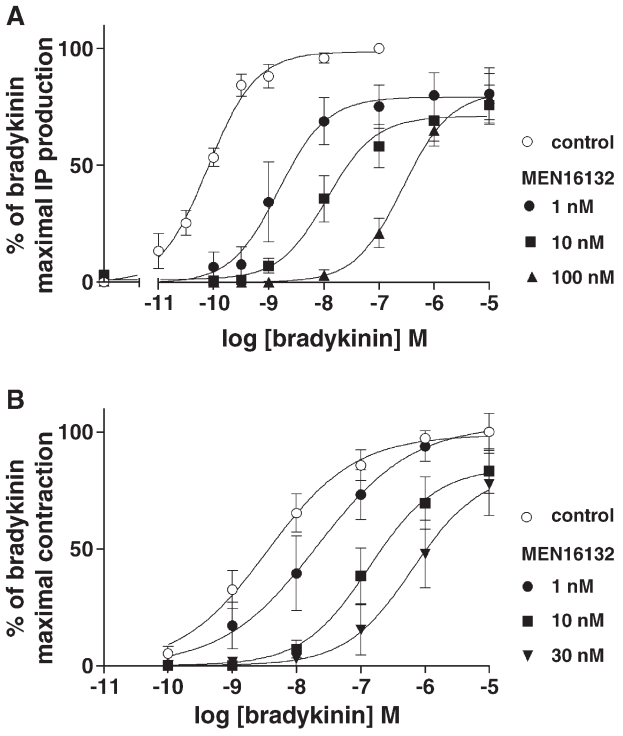

Fig. 4. Functional antagonism of MEN16132 at the human bradykinin B_2 receptor. A: Concentration–response curves to bradykinin on IP production in CHO cells expressing the human bradykinin B_2 receptor in the absence (control) and presence of the indicated concentration of MEN16132, preincubated 15 min before the agonist response. Values are the mean±S.E.M. from 3–4 independent experiments performed in triplicate. B: Concentration–response curves to bradykinin in the human detrusor smooth muscle in the absence (control) and presence of the indicated concentration of MEN16132, preincubated 15 min before the agonist response. Data points are the mean±S.E.M. of at least 5 experiments.

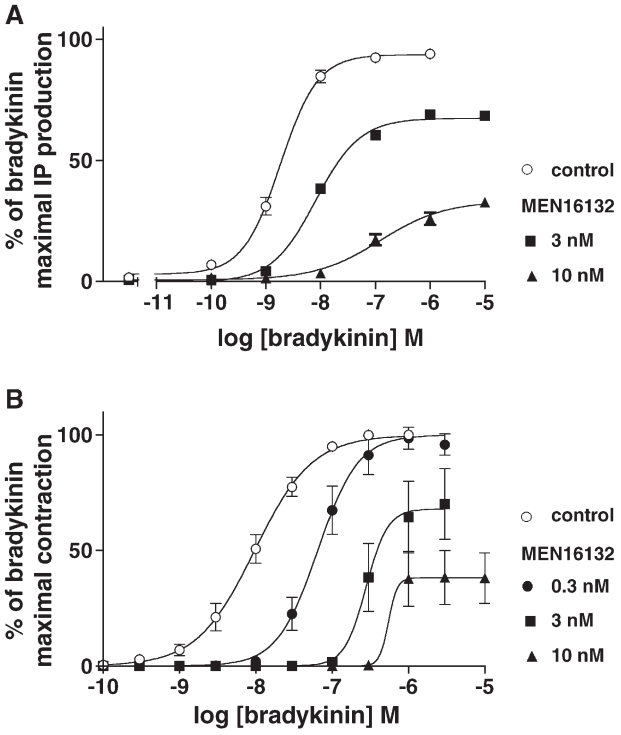

Fig. 5. Functional antagonism of MEN16132 at the guinea pig bradykinin B_2 receptor. A: Concentration–response curves to bradykinin on IP production in cultured colonic myocytes in the absence (control) and presence of the indicated concentration of MEN16132, preincubated 15 min before the agonist response. Values are the means±S.E.M. from 3–4 independent experiments performed in triplicate. B: Concentration–response curves to bradykinin in the longitudinal ileum smooth muscle in the absence (control) and presence of the indicated concentration of MEN16132, preincubated 15 min before the agonist response. Data points are the mean±S.E.M. of at least 5 experiments.

3.2.2. Antagonist potency at the guinea pig B_2 receptor

In cultured guinea pig colonic myocytes bradykinin (0.1 – 1000 nM) concentration-dependently increased the IP production, with a maximal increase of 17±3 fold over the basal. MEN16132 did not affect the basal IP accumulation at any of the concentrations used, and produced a concentration-dependent rightward shift of the agonist concentration–response curve, reducing also its E_{max}. In the presence of 3 and 10 nM antagonist concentration bradykinin produced E_{max} was 72±1 and 38±2% of control response, respectively (Fig. 5A). The calculated apparent pK_B value was 9.8 (9.7–9.9, 95% c.l).

In the guinea pig longitudinal smooth muscle preparation bradykinin produced a concentration-dependent contraction (Fig. 5B). Whereas MEN16132 neither showed agonist effect up to the highest concentration used, nor affected the KCl (80 mM) induced responses, it produced a concentration-dependent (0.3–30 nM) reduction of bradykinin induced E_{max} (Fig. 5B). The calculated apparent pK_B value was 10.1 (9.8–10.4, 95% C.I.).

In the same preparation the reversibility of the MEN16132 receptor blockade was evaluated as the capability of the smooth muscle to recover the contraction produced by a single

Table 2
Binding affinity of MEN16132 at wild type and mutant human bradykinin B_2 receptors

[^{3}H]bradykinin binding			
Human bradykinin B_2 receptor	TM	K_i [nM] (95% C.I.) MEN16132	F_{mut}
Wild type		0.09 (0.07–0.11)	*1*
E47A	1	0.15 (0.12–0.18)	
D76A	2	0.06 (0.049–0.068)	
T89A		0.077 (0.057–0.10)	
I110A	3	28 (19–41)[a]	*311*
S111A		0.11 (0.085–0.14)	
N113A		0.37 (0.31–0.45)[a]	*4*
L114A		0.10 (0.09–0.11)	
S117A		0.15 (0.11–0.20)	
T158A	4	0.09 (0.075–0.11)	
M165T		0.11 (0.08–0.14)	
L166F		0.11 (0.09–0.14)	
T197A	5	0.11 (0.084–0.15)	
S211A		0.11 (0.084–0.14)	
F252A	6	0.18 (0.15–0.23)	
W256A		0.44 (0.37–0.51)[a]	*5*
Q288A	7	0.15 (0.10–0.21)	
S291A		0.11 (0.086–0.13)	
F292A		0.22 (0.17–0.29)	
Y295F		9.0 (7.9–10.5)[a]	*100*
Y295A		68 (56–83)[a]	*755*
Y295W		2.7 (2.4–3.5)[a]	*30*
W256A/Y295F	6/7	151 (116–197)[a]	*1670*

Experiments were carried out on membrane preparations from pooled clones of CHO cells stably expressing the wild type or mutant human B_2 receptors, by using the peptide agonist [^{3}H]bradykinin as radioligand. K_i values of MEN16132 were calculated according to the concentration and K_d of [^{3}H]BK for each mutant receptor (See Methods). F_{mut} index was calculated as K_i(mutant receptor)/K_i(wild type receptor), and corresponds to fold decrease in affinity.
TM, receptor transmembrane segment.
Data are from 3 independent experiments, each one performed in duplicate.
[a] Statistically significant differences in terms of ligand affinity values at the mutant receptors were postulated on the basis of non overlapping 95% C.I. and F_{mut} index greater than 3.

concentration of bradykinin (100 nM). After a 15 min contact time with MEN16132 (1 nM), bradykinin response was 49 ±10% of the matched control response. After 30, 60, and 90 min washout (see methods) bradykinin response was 71±6%, 68 ±8%, and 85±6%, of time-matched control response, respectively.

3.3. Evaluation of receptor discriminants participating in MEN16132 binding site: affinity at the wild type and point-mutated human bradykinin B_2 receptor

The affinity of MEN16132 was determined at 24 different point-mutated human bradykinin B_2 receptors stably transfected in CHO cells, using cell membrane preparations and the agonist [^{3}H]bradykinin (Table 2) or antagonist [^{3}H]MEN11270 (Table 3) radioligands.

All mutations were produced at level of transmembrane (TM) portions of the human bradykinin B_2 receptor sequence, and the peptide agonist bradykinin could bind most of the mutant receptors, with the exception of W86A and F259A (TM 2 and 6, respectively) (see below).

The bradykinin affinity determined by means of homologous competitive binding experiments (pK_d at the wild type human bradykinin B_2 receptor was 9.3, 9.2–9.4, 95% C.I.) did not significantly change at the bradykinin B_2 receptor mutants studied (Meini et al., 2002, 2004; Bellucci et al., 2003).

MEN16132 competed for the [^{3}H]bradykinin binding site at the wild type B_2 receptor with a pK_i value of 10 (9.9–10.15, 95% C.I.), and no differences in affinity could be observed at the following mutated receptors: E47A (TM1), D76A, T89A (TM2), S111A, L114A, S117A (TM3), T158A, M165T, L166F (TM4), T197A, S211A (TM5), F252A (TM6), Q288A, S291A, and F292A (TM7) (Table 2). The TM3 substitution that caused the greater reduction of MEN16132 affinity (300-fold), was the I110A mutant (pK_i 7.5, 7.4–7.7, 95% C.I.), whereas a smaller but significant decrease was obtained at the N113A (4-fold; pK_i 9.4, 9.3–9.5, 95% c.l) mutant. The alanine mutation of the W256 residue in TM6 produced a 5-fold reduction of MEN16132 affinity (pK_i value 9.3, 9.2–9.4, 95% C.I.). The Y295 residue located in TM7 was changed to produce three different mutants: Y295A, Y295F and Y295W. All these three substitutions caused a loss in affinity for MEN16132 but at different extents: 700-fold at the Y295A (pK_i 7.15, 7.1–7.3, 95% C.I.), 100-fold at the Y295F (pK_i 8.0, 7.9–8.1, 95% C.I.), and 30-fold at the Y295W (pK_i 8.5, 8.4–8.6, 95% C.I.) mutant. Moreover, the double mutation W256A/Y295F impaired MEN16132 affinity by 1670-fold (pK_i value 6.8, 6.7–6.9, 95% C.I.) (Table 2).

MEN16132 affinities at W86A(TM2) and F259A (TM6) mutants were studied by means of inhibition experiments at the antagonist [^{3}H]MEN11270 binding site. Homologous competition binding experiments did not reveal any significant changes for MEN11270 affinity at these mutants, as compared with the wild type receptor (pK_i 9.0, 8.9–9.1, 95% C.I.) (Meini et al., 2002). MEN16132 competed with high affinity for the

Table 3
Binding affinity of MEN16132 at wild type and mutant human bradykinin B_2 receptors

[^{3}H]MEN11270 binding			
Human bradykinin B_2 receptor	TM	K_i [nM] (95% C.I.) MEN16132	F_{mut}
Wild type		0.6 (0.4–0.8)	1
W86A	2	717 (450–1140)[a]	1195
F259A	6	0.3 (0.2–0.5)	

Experiments were carried out on membrane preparations from pooled clones of CHO cells stably expressing the wild type or mutant human bradykinin B_2 receptors, by using the peptide antagonist [^{3}H]MEN11270 as radioligand. K_i values of MEN16132 were calculated from heterologous inhibition experiments according to the concentration and K_d of [^{3}H]MEN11270 for each mutant receptor (See Methods). F_{mut} index was calculated as K_i(mutant receptor)/K_i (wild type receptor), and corresponds to fold decrease in affinity.
TM, receptor transmembrane segment.
Data are from 3 independent experiments, each one performed in duplicate.
[a] Significantly different from wild type ($P<0.05$).

[^{3}H]MEN11270 binding site both at the wild type (pK_i 9.2, 9.1–9.3, 95% C.I.) and F259A (pK_i 9.5, 9.3–9.8, 95% C.I.) receptors. On the contrary, a 1300-fold decrease in MEN16132 affinity value was measured at the W86A mutant (pK_i 6.1, 5.9–6.3, 95% C.I.) (Table 3).

4. Discussion

The present study shows the pharmacological profile of MEN16132 as a novel bradykinin B_2 receptor selective non-peptide antagonist, endowed with subnanomolar affinity for the human B_2 receptor, micromolar affinity for the human B_1 subtype, and devoid of unspecific effects at several other receptors or channels.

In the field of bradykinin B_2 receptor antagonists pharmacology a great effort has addressed the difference in affinity values measured in binding versus functional assay (Burkard et al., 1996; Wieczorek et al., 1997). This peculiar pattern has been defined "binding paradox" (Hall, 1992), and explained by the different ionic composition of the buffers used in the different assays (Ransom et al., 1992; Paquet et al., 1999). In CHO cells stably expressing the human bradykinin B_2 receptor, we have previously characterized the antagonism of both peptide and nonpeptide antagonist ligands, and for all of them we reported a similar antagonist affinity (pK_B ranging between 8.4 and 8.8) and a competitive antagonism (Meini et al., 2004; Bellucci et al., 2004). From the present data it appears that MEN16132 is not subjected to the same molecular mechanisms which differently bias the estimate of affinity of other B_2 antagonist ligands. In fact, MEN16132 antagonized the IP accumulation induced by bradykinin with an apparent affinity (pK_B 10.1) only 2-fold lower than its affinity determined at [^{3}H]bradykinin binding site (pK_i 10.5) in CHO cells expressing the human bradykinin B_2 receptor. Moreover, we present evidence that MEN16132 exerts a similar competitive antagonism on bradykinin-induced contractile responses of the human detrusor smooth muscle with an affinity (pK_B 9.9) 10–30-fold higher than that measured for Icatibant (pK_B 8.4), MEN11270 (pK_B 8.4), or FR173657 (pK_B 8.6) (Meini et al., 2000a) in the same assay, or even LF16-0687 (pK_B 9.1), as measured in another classical human B_2 receptor assay, i.e., the human umbilical vein (Gobeil et al., 1996; Pruneau et al., 1999).

The affinity and potency of MEN16132 was evaluated also for the guinea pig bradykinin B_2 receptor: inhibition studies of [^{3}H]bradykinin binding in airways and ileum tissue membrane preparations indicate that MEN16132 (which exhibits subnanomolar affinity) is as potent as peptide antagonists, but significantly more potent than the nonpeptides LF16-0687 (3-fold) and FR173657 (30–50-fold). Again the high affinity of MEN16132 well matches its high potency in functional smooth muscle contractility studies (guinea pig ileum): MEN16132 behaved as an insurmountable antagonist endowed with high potency (pK_B 10.1) but still able to reverse from the receptor after the blockade. We previously reported the analysis of Icatibant, MEN11270, and FR173657 antagonism on the BK induced smooth muscle contraction of guinea pig longitudinal ileum (Meini et al., 2000b), as a classical bioassay for B_2 receptor pharmacology (Hall, 1992). A diverse antagonist behaviour was highlighted by these compounds under comparable experimental conditions, which indicated Icatibant and FR173657 as insurmountable antagonists (pK_B 9.5 and 9.2, respectively), and MEN11270 as a competitive antagonist (pK_B 8.3) (Meini et al., 2000b), as previously reported also for LF16-0687 (pK_B 9.1, Pruneau et al., 1999). In order to check if differences in antagonism type could be related to the different assay (IP production versus smooth muscle contractility), we tested MEN16132 and the reference antagonists in a model of cultured guinea pig colonic myocytes. In agreement with the above reported results, MEN16132 displayed a subnanomolar affinity in inhibiting [^{3}H]bradykinin binding to cell membranes (pK_i 10.3), and an insurmountable antagonism towards bradykinin-induced IP accumulation (pK_B 9.8). Accordingly, in this cell assay FR173657 depressed by 75% the agonist E_{max}, whereas LF16-0687 did not (pA_2 9.6 and 9.1, respectively, as determined at 100 nM antagonist concentration, data not shown), indicating that nonpeptide B_2 receptor antagonists may be distinct for their antagonism at the guinea pig B_2 receptor. In other words, these results suggest that MEN16132 and FR173657 find, in the guinea pig B_2 receptor sequence, a structural counterpart which may be responsible for a different kinetic of the ligand–receptor interaction.

In the G protein-coupled receptors the ligand binding crevice of small molecules is usually located in the hydrophobic core comprised amongst TMs 3, 6, and 7, whose motions appear to be important for receptor activation (Gether and Kobilka, 1998). Indeed the binding site of nonpeptidic B_2 receptor antagonist ligands at the human bradykinin B_2 receptor, mainly involves residues belonging to these TM sequences (Marie et al., 1999, 2001; Meini et al., 2002, 2004). Our results with Ala mutations at E47 (TM1), D76, T89 (TM2), S111, N113, L114 (TM3), T158 (TM4), T197, S211 (TM5), and S291 (TM7) indicate that these residues do not participate in the high affinity interaction of MEN16132 to the human bradykinin B_2 receptor, comparably to data presented for the two antagonists FR173657 and LF16-0687 (Meini et al., 2002; data not shown for E47A). Similarly to these nonpeptide ligands, a significant reduction in affinity for MEN16132 was observed at the W86A (TM2) but not at the F259A (TM6) mutant receptors, which were previously reported to abrogate the binding of the agonist bradykinin (Leeb et al., 1997; Meini et al., 2002). In line with previous data obtained with the two reference nonpeptide antagonists, MEN16132 binding site should be comprised among TM2 (W86A), TM3 (I110A), TM6 (W256A), and TM7 (Y295A and Y295F), as highlighted by the reduction in MEN16132 affinity exerted by the mutations at the residues studied.

Contrary to what has been observed for other nonpeptides (Marie et al., 2001; Meini et al., 2002), a participation for the N113 residue, which lies one helical turn down from I110 and is involved in the maintainance of the receptor inactive conformation (Marie et al., 1999), could be hypothesized for MEN16132 binding. Moreover, whereas the loss of affinity of MEN16132 for the I110A mutant was in a similar order of magnitude than that measured with LF16-0687 and FR173657

(300-fold, Meini et al., 2004), a different pattern was observed at mutations of the Y295 residue. In agreement with data obtained with LF16-0687, and contrary to what observed for FR173657 (Marie et al., 2001; Meini et al., 2002), MEN16132 affinity was progressively reduced by the Y295F (100-fold) and Y295A (733-fold) mutations, suggesting a role for both the hydroxyl and phenyl moieties of the Y295 residue in the receptor recognition site of MEN16132. As a confirm, the substitution of the Y295 residue with a Trp residue (Y295W), which maintains the capability to form hydrogen bonds, hampered the MEN16132-receptor complex at a less extent (30-fold).

In respect with MEN16132 binding site, a further analogy and difference as compared to the LF16-0687 and FR173657 compounds, respectively (Meini et al., 2002), was the impairment of its affinity (5-fold) at the W256A mutant. This residue lies close to I110 and Y295, which are part of a network of residues which have been hypothesized to control the balance between active and inactive conformations of the human B_2 receptor (Marie et al., 2001). The mutual participation of both W256 and Y295 residues in the MEN16132-B_2 receptor interaction is supported by the greater reduction (1670-fold) in the ligand affinity at the double mutant W256A/Y295F, thus confirming that both mutated residues belong to the same receptor binding crevice.

So far it appears, in agreement with chemical relationship, that MEN16132 binding site is common to that of both LF16-0687 and FR173657 ligands, although the binding outline at receptors bearing aminoacidic substitutions in TM6 (W256A) and TM7 (Y295A, Y295F), suggests a greater similarity with LF16-0687. Whether or not these differences may account for the greater potency and affinity by MEN16132 over the other antagonists remains a speculative hypothesis at present.

Bradykinin B_2 receptor antagonists have been proposed as potential therapeutic agents for a variety of inflammatory diseases, such as asthma and rhinitis, brain injury, and hereditary angioedema (Akbary et al., 1996; Rosenkranz et al., 2005; Sobey, 2003; Turner et al., 2001). As a whole the present work shows the molecular and pharmacological profile of MEN16132 as a novel bradykinin B_2 receptor selective nonpeptide antagonist, which is at least 10-fold more potent than the other previously presented antagonists. Evidence supporting the pharmacological profile of MEN16132 as a potent and long lasting B_2 receptor antagonist in in vivo animal models has been very recently provided (Valenti et al., 2005).

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