

RESEARCH PAPER

Comparison of the molecular interactions of two antagonists, MEN16132 or icatibant, at the human kinin B₂ receptor

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BACKGROUND AND PURPOSE

Icatibant is a well-known kinin B₂ receptor antagonist currently used for angiooedema attacks. MEN16132 is a non-peptide B₂ receptor antagonist, more potent and long lasting than icatibant in different models. Here we studied the reasons for these differences between the two antagonists.

EXPERIMENTAL APPROACH

Rate of reversibility (over about 3 h) of the functional receptor blockade exerted by the antagonists was compared (inositol phosphates accumulation assay) in CHO cells expressing the human B₂ receptor and in human synovial cells. Antagonist pretreated cells were washed with medium and the time taken to restore bradykinin (BK) response measured. Antagonist affinity was measured by radioligand binding to wild type and mutated B₂ receptors.

KEY RESULTS

Recovery of BK-induced responses was slower in cells pretreated with MEN16132 than in those treated with icatibant. The affinity of icatibant (for the [³H]-BK or the B₂ receptor antagonist [³H]-MEN11270 binding site) was compared to that of MEN16132 using a panel of point-mutated receptors with mutations located at the transmembrane regions of the B₂ receptor, previously shown to decrease MEN16132 high affinity interaction. No consistent decrease of icatibant affinity was observed. From the different affinity of MEN16132 derivatives at wild type and W86A (transmembrane 2 region) receptors, and by evaluating its antagonist profile at the D266A/D284A double mutant receptor, a model of the MEN16132-B₂ receptor complex is proposed.

CONCLUSIONS AND IMPLICATIONS

MEN16132 dissociated from the B₂ receptor compartment more slowly than icatibant and interacted at a deeper level in transmembrane regions of the receptor.

Abbreviations

BK, bradykinin; DMSO, dimethyl sulfoxide; GPCR, G protein-coupled receptor; IP, inositol phosphate; PBS, phosphate buffered saline; TM, transmembrane

Introduction

Bradykinin (BK, Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is a small peptide ubiquitously formed by the action of kallikreins

on circulating kininogens (Regoli and Barabé, 1980). Once formed it acts on the B₂ receptor (receptor nomenclature follows Alexander *et al.*, 2009), a transmembrane (TM) G protein-coupled receptor (GPCR), which has been

pharmacologically characterized and molecularly cloned. Activation of the B₂ receptor leads to G_{q/11} protein coupling, which triggers phospholipase C with consequent inositol 3-phosphate and diacylglycerol formation, and release of intracellular calcium, and all the consequently activated intracellular pathways (Leeb-Lundberg *et al.*, 2005 for review).

The participation of BK in inflammation and pain, extensively shown in different models, drove the search to discover selective kinin B₂ receptor antagonists, of peptide structure first and then non-peptide. Icatibant (formerly named Hoe140, D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK, Hock *et al.*, 1991; Wirth *et al.*, 1991) is now the most investigated B₂ receptor antagonist, and the only one to reach the market as therapy, for hereditary angioedema attacks (Firazyr®). Its structure was designed to confer metabolic resistance to peptidases and rigidity at the C-terminal portion of the peptide backbone, features that have been reported to contribute to its metabolic resistance and prolonged duration of action in different animal models (Wirth *et al.*, 1991; Bond *et al.*, 1992; Legat *et al.*, 1994). Following this, several non-peptide B₂ receptor antagonists have been synthesised (Dziadulewicz, 2005; Fincham *et al.*, 2009). MEN16132 is a non-peptide antagonist selective for the B₂ receptor. It displays a remarkably high affinity and antagonist potency in different species (human, guinea pig, mouse, rat, rabbit and pig) as shown by radioligand binding (using tritiated BK as radioligand) and functional *in vitro* assays [inositol phosphate (IP) accumulation and smooth muscle contractility] (Cucchi *et al.*, 2005; Meini *et al.*, 2007; 2009; 2010). Furthermore, in preclinical models of inflammation and pain, MEN16132 was effective and long lasting in blocking both exogenously administered and endogenously formed BK (Valenti *et al.*, 2005; 2008; Cialdai *et al.*, 2009). In all these models the pharmacological properties of MEN16132 were compared with those of icatibant, and despite their comparable high affinity for the B₂ receptor, MEN16132 displayed a significantly greater antagonistic potency and longer duration of action both *in vitro* and *in vivo*.

The present investigation deals with the reasons for these clear-cut and constant differences between MEN16132 and icatibant. First, the reversibility of antagonist action in blocking B₂ receptors was compared through functional experiments (IP accumulation assay) both at the recombinant human B₂ receptor (stably expressed in CHO cells) and at the native B₂ receptor (endogenously expressed in human fibroblast-like synovial cells). These two cell assays were chosen in order to gain information on the antagonistic interaction with the human B₂ receptor: CHO cells as the system used to investigate the antagonistic interaction mode, synovial cells as a likely therapeutic cell target (MEN16132 is currently in phase II as intraarticular treatment for osteoarthritis, and synovitis is one of the main aspect of this disease). Then, through radioligand binding experiments the affinities of MEN16132 and icatibant were compared, using a panel of point-mutated receptors, with mutated amino acid residues located at TM level of the human B₂ receptor sequence. Last, in order to propose and refine a docking model of MEN16132 with the human B₂ receptor, its affinity outline, in comparison with a series of modified derivatives, and its antagonist profile were investigated at some mutated receptors.

Overall, our data indicate that MEN16132 is more potent than icatibant in regard to its slower reversibility from the B₂

receptor compartment. This is, in turn, due to a ligand-receptor interaction located in the TM portion of the receptor protein, and involving residues implied in receptor activation.

Methods

Cells

Dihydrofolate reductase (DHFR)-deficient CHO cell line CHO DUKX-B11 stably expressing the wild type or mutated human kinin B₂ receptors (Bellucci *et al.*, 2003; Meini *et al.*, 2004) were cultured in Iscove's modified Dulbecco's medium (IMDM, Cat. No. I3390, Sigma-Aldrich, Dorset, UK), containing 10% dialysed foetal bovine serum (FBS, Cat. No. SH30079.03, Hyclone, UT, USA), 2 mM L-glutamine (Gln, Cat. No. G7513, Sigma-Aldrich, Dorset, UK) and 1% of penicillin and streptomycin (Cat. No. P4333, Sigma-Aldrich). The cells were subcultured by using 0.25% trypsin and 1 mM EDTA to detach them, and then cultured in 175 cm² flasks and maintained in a humidified atmosphere at 37°C with 5% CO₂. Cells at confluence were used to prepare membranes (see below) for radioligand binding experiments, or seeded in 75 cm² flasks (7000 cells·cm⁻²) for cell culture maintenance and membrane preparation, and in 24-well plates (150 000 cells·cm⁻²) for functional experiments.

Human fibroblast-like synoviocytes derived from normal synovial tissue were purchased as frozen vials (catalogue no. 408-05a: lots no. 1828 and 1798) from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were handled as recommended by the producer: the seeding density for attachment was 7000 cells·cm⁻² on plastic wells, and the culture medium was Synoviocyte Growth Medium (ECACC, catalogue no. 06091516, CAI no. 415-500), with glutamine (2 mM), penicillin (50 µg·mL⁻¹), streptomycin (50 µg·mL⁻¹) and amphotericin B (15 µg·mL⁻¹). When cells reached 90% of confluency they were split by using a subculture reagent kit according to the manufacturer's instructions (Lonza, catalogue no. CC3233, Walkersville, MD, USA) and seeded in 75 cm² flasks (7000 cells·cm⁻²) for cell culture maintenance and membrane preparation, and in 24-well plates (25 000 cells·cm⁻²) for functional experiments. Cells were used between 2 and 10 passages.

Measurement of IP accumulation

Cells were grown in 24-well tissue culture plates and labelled with myo-[³H] inositol (0.5 mL·well⁻¹, 1 µCi·mL⁻¹) in Iscove's modified Dulbecco's medium and Ham's F12 medium (1:1) containing 1% FBS and L-glutamine (2 mM). After 24 h (CHO cells) or 48 h (human synoviocytes) the medium was removed and cells were preincubated for 15 min (37°C) with antagonists at the indicated concentration in the stimulation buffer: phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺ (135 mM), with HEPES (20 mM), CaCl₂ (2 mM), MgSO₄ (1.2 mM), EGTA (1 mM), glucose (11.1 mM), captopril (10 µM), bovine serum albumin (BSA) (0.05%), LiCl (25 mM). In reversibility experiments BK (3 nM) was added after different times during which the medium was totally renewed, both in vehicle (control) and antagonist-treated cells, and reaction stopped after 60 min agonist incubation period. In

the experiments in which stimulation was performed with the non-peptide B₂ receptor agonist FR190997, an incubation period of 30 min was used as previously described (Bellucci *et al.*, 2003; 2004). Total IP levels were determined as previously described (Bellucci *et al.*, 2003). Determinations were performed in triplicate.

Membrane preparation

Cells at confluence were washed out of the medium by PBS without calcium and magnesium and harvested by incubation at 37°C with Hanks Buffered Salt Solution (HBSS, pH 7.4) HEPES (10 mM), EDTA 1 mM, and a cocktail of peptidase inhibitors: 1,10 phenanthroline (1 mM), EGTA (1 mM), captopril, leupeptin, soybean trypsin inhibitor, DL-2-mercaptomethyl-3-guanidoethylthiopropionic acid (MERGETPA; 1 µM each), chymostatin (3.3 µM), phenylmethyl-sulphonyl fluoride (0.1 mM) and bacitracin (140 µg·mL⁻¹). Cells were then washed in N-tris [hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES, 10 mM, pH 7.4, at 4°C), containing the cocktail of peptidase inhibitors described above and homogenized with a Polytron (PT 3000, Kinematica), set at 15 000 r.p.m. for 30 s. The homogenate was centrifuged at 45 000× *g* for 45 min (4°C). The protein concentration was determined by the method of Bradford using a Bio-Rad kit and BSA as reference standard. The pellet was resuspended to obtain 7.5 mg·mL⁻¹ membrane protein concentration and frozen in 1 mL aliquots by immersion in liquid nitrogen, and then stored at -80°C until use.

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

Radioligand binding

The buffer used for binding experiments was TES (10 mM, pH 7.4) containing 1,10-phenanthroline (1 mM), bacitracin (140 µg·mL⁻¹), and BSA (1 g·L⁻¹). Binding assay was performed in a final volume of 0.5 mL. An incubation time of 60 min at room temperature was used. Competition binding studies were carried out with a radioligand concentration comparable with the calculated K_d value (0.1–0.2 nM [³H]-BK or [³H]-MEN11270), which did not differ among the studied receptors. At this concentration, bound ligand was less than 10% of the total added radioligand concentration. In preliminary experiments we observed that the specific binding of each radioligand was directly proportional to membrane concentration. A membrane protein concentration between 90 and 200 µg·mL⁻¹ was used for each receptor to obtain a signal of 1500–3000 dpm·assay⁻¹ of specific binding. Non-specific binding was defined as the amount of labelled radioligand bound in the presence of 1 µM of unlabelled appropriate ligand. Under each selected condition the specific binding represented approximately 70–80% of the total binding. Maximal receptor density (B_{max}) was evaluated by using three different saturating concentrations of [³H]-BK (5, 10 and 20 nM).

Competing ligands were tested in a wide range of concentrations (1 pM–1 µM). Each experiment was performed in duplicate. Experiments were designed to minimize experimental differences, that is, different compounds were simultaneously tested on different cell systems or receptors (wild

type and mutants or CHO and synovial cell membranes). Competing ligands were placed into 96-well plates by the aid of a liquid handling automation system (MultiProbe II, PerkinElmer, Waltham, MA, USA). All incubations were terminated by rapid filtration through UniFilter-96 plates (PerkinElmer), pre-soaked for at least 2 h in polyethylenimine (PEI) 0.6%, and using a MicroMate 96 Cell Harvester (PerkinElmer). 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 (PerkinElmer), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (PerkinElmer).

Analysis of pharmacological data

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

Binding data were fitted by using GraphPad Prism 4.0 in order to determine the equilibrium dissociation constant (K_d) from homologous competition experiments, the ligand concentration inhibiting the radioligand binding of the 50% (IC₅₀) from heterologous competition experiments. K_i values were calculated from IC₅₀ using the Cheng-Prusoff equation ($K_i = IC_{50}/(1 + [radioligand]/K_d)$) according to the concentration and K_d of the used radioligand (Cheng and Prusoff, 1973). B_{max} values were expressed as [³H]-BK specifically bound as fmol·mg⁻¹ of protein.

Concentration–response curves in functional experiments were analysed by sigmoidal non-linear regression fit using the GraphPad Prism 4.0 program (San Diego, CA, USA) to determine the molar concentration of the agonist producing the 50% (EC₅₀) of its maximal effect (E_{max}). Agonist responses obtained either in the absence (control) or presence of antagonist were normalized towards the E_{max} of control agonist response. The antagonist potency was quantified as pA₂ values, defined as the molar concentration of antagonist that produces a twofold shift to the right of the agonist concentration–response curve, as follows: $pA_2 = \log [CR - 1] - \log [\text{antagonist concentration}]$, where CR is the ratio of equi-active concentrations of agonist in the presence and absence of antagonist (Kenakin, 1997).

Modelling

The human B₂ receptor model was obtained as previously described (Meini *et al.*, 2002; 2004). Briefly, the starting point was the crystallographic structure of bovine rhodopsin receptor (Palczewski *et al.*, 2000), further refined taking into account the recent crystal structure of the β₂-adrenoceptor (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007). Residues were mutated according to the proper human B₂ receptor sequence (Hess *et al.*, 1992) and their side chains positions refined. Then an analysis was performed on receptor amino acid residues within the TM segments, and presumed facing inward, by means of the GRID program (Molecular Discovery Ltd, London, UK) with water, hydrophobic and amphiphilic probes, thus defining lipophilic pockets: a larger one among the TM helices 3, 4, 5 and 6, and a smaller one among the TM helices 2, 3 and 7. These two pockets were considered and evaluated as potential binding sites for MEN16132. Only the largest pocket would allow a

meaningful docking of MEN16132. A manual docking was then applied to a relaxed conformation of the ligand, using Tripos Sybyl software (v. 8.0 Tripos, St. Louis, MO, USA). The docking was based on the results from the mutational analyses, with some slight adjustment of bond torsions to fit the best orientation of the ligand.

Materials

[³H]-BK (specific activity 90 Ci·mmol⁻¹) and *myo*-[1,2-³H] inositol (specific activity 60–80 Ci·mmol⁻¹) were provided by Perkin Elmer, and [³H]-MEN11270 (specific activity 30 Ci·mmol⁻¹) (H-DArg-Arg-Pro-Hyp-Gly-Thi-c(Dab-DTic-Oic-Arg)c(7γ-10α); Meini *et al.* 1999) was synthesized by SibTech Inc. (Newington, CT, USA). The peptide B₂ receptor agonist BK was obtained from PolyPeptide (Strasbourg, France). DL-2-mercaptomethyl-3-guanidoethylthiopropionic acid (MERGETPA) was from Merck (Darmstadt, Germany), whereas all other enzyme inhibitors and salts were from Sigma (Dorset, UK). MEN16132 ((4-(S-amino-5-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl)phenylsulfonamido]-tetrahydro-2H-4-pyranylcarbonyl}piperazino)-5-oxopentyl](trimethyl) ammonium chloride hydrochloride), MEN15880, MEN16298, MEN16303, MEN16318 (structures in Table 1) and icatibant (DArg[Hyp³, Thi⁵, DTic⁷, Oic⁸]BK) were synthesized in Menarini Ricerche, dissolved in distilled water at 10 mM and stored at –25°C, and further dilutions were made in the appropriate assay buffer.

The non-peptide B₂ receptor agonist FR190997 (8-[2,6-dichloro-3-[(N-(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino] benzyloxy]-2-methyl-4-(2-pyridylmethoxy) quinoline, Aramori *et al.* 1997), kindly provided by Fujisawa company (now Astellas), was dissolved in DMSO up to a concentration of 100 μM.

Results

MEN16132 and icatibant affinity for [³H]-BK specifically bound to the recombinant and endogenously expressed human B₂ receptor

The affinity of MEN16132 and icatibant was assessed at the recombinant and endogenously expressed human B₂ receptor by using [³H]-BK as radioligand and membranes prepared from CHO cells stably expressing the human B₂ receptor and human fibroblast-like synoviocytes (Figure 1) respectively. Homologous displacement of BK gave K_d values of 0.16 nM (0.095–0.28, 95% c.i.) and 0.20 nM (0.11–0.35, 95% c.i.) with membranes of CHO cells and synoviocytes respectively. B_{max} values were 322 ± 14 and 553 ± 51 fmol·mg⁻¹ proteins in CHO and synovial cells respectively (*n* = 3).

Both antagonists totally inhibited the [³H]-BK specific binding in a concentration-dependent manner, and the resulting inhibition curves were overlapping in both cell membrane systems. From experiments performed with CHO

Table 1

Comparison of icatibant and MEN16132 affinity at wild type and mutant human B₂ receptors (hB₂R)

hB ₂ R	TM	MEN16132 K _i [nM] (95% CI)	[³ H]-bradykinin binding		
			F _{mut}	icatibant K _i [nM] (95% CI)	F _{mut}
Wild type		0.09 (0.07–0.10)	1	0.08 (0.07–0.09)	1
E47A	1	0.15 (0.12–0.18)	1.6	0.19 (0.16–0.25)	2.4
I110A	3	28 (19–41)	311	0.25 (0.22–0.39)	3.1
S111A	3	0.11 (0.085–0.14)	1.2	0.16 (0.09–0.31)	2
L114A	3	0.10 (0.09–0.11)	1.1	0.11 (0.08–0.13)	1.4
S117A	3	0.15 (0.11–0.20)	1.7	0.19 (0.14–0.23)	2.3
T158A	4	0.09 (0.075–0.11)	1	0.12 (0.06–0.22)	1.5
L166F	4	0.11 (0.09–0.14)	1.2	0.15 (0.12–0.20)	1.8
F252A	6	0.18 (0.15–0.23)	2	0.02 (0.01–0.05)	0.2
W256A	6	0.44 (0.37–0.51)	5	0.16 (0.14–0.20)	2
Q288A	7	0.15 (0.10–0.21)	1.7	0.25 (0.19–0.40)	3.1
F292A	7	0.22 (0.17–0.29)	2.4	0.11 (0.12–0.25)	1.4
Y295F	7	9.0 (7.9–10.5)	100	0.39 (0.31–0.51)	4.9
Y295A	7	68 (56–83)	755	0.15 (0.06–0.38)	1.9
Y295W	7	2.7 (2.4–3.5)	30	0.07 (0.04–0.15)	0.9
W256A/Y295F	6/7	151 (116–197)	1670	0.35 (0.17–0.71)	4.4

The affinity was evaluated at the peptide agonist [³H]-bradykinin radioligand binding site. F_{mut} index was calculated as K_i (mutant receptor)/K_i (wild type receptor), and corresponds to fold decrease in affinity. F_{mut} indexes greater than 3 are shown in bold type. TM indicates the transmembrane portion of the receptor where the mutated amino acid residue is located. Data are from three independent experiments, each one performed in duplicate. K_i values measured for MEN16132 were previously presented (Cucchi *et al.*, 2005).

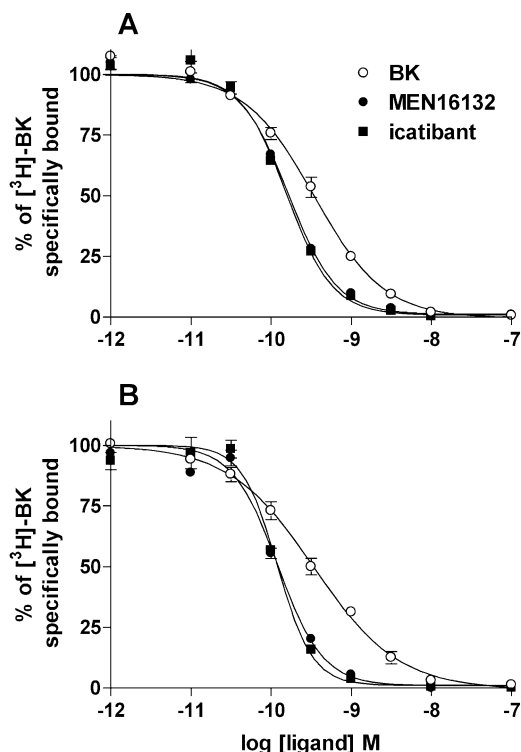


Figure 1

MEN16132 and icatibant affinity at the [³H]-bradykinin (BK) binding site in CHO cells expressing the human B₂ receptor (A) and in human fibroblast-like synovial cells (B).

cell membranes the calculated K_i values were 0.087 nM (0.078–0.092, 95% c.i.) for MEN16132 and 0.081 nM (0.071–0.092, 95% c.i.) for icatibant (Figure 1A), and from those performed with synoviocyte membranes, K_i values were 0.067 nM (0.059–0.076, 95% c.i.) for MEN16132 and 0.066 nM (0.058–0.075, 95% c.i.) for icatibant (Figure 1B).

Reversibility pattern of B₂ receptor functional blockade exerted by MEN16132 and icatibant in CHO cells expressing the human B₂ receptor and in human synovial cells

The reversibility of MEN16132 and icatibant from receptor compartment was investigated in CHO cells expressing the human B₂ receptor and in human synovial cells.

Agonist responses obtained with antagonist-treated cells were expressed as a percentage of the time-matched control response, that is, BK-induced IP response obtained in cells not treated with antagonists and that were given the same number of washing cycles.

In both assays cells were preincubated for 15 min with different concentrations of antagonists which inhibited the BK (3 nM) induced IP accumulation.

In CHO cells, MEN16132 at 1 nM concentration inhibited the BK-induced IP accumulation by 53%, whereas at higher concentrations (10, 100 nM and 1 μM, Figure 2A) it produced a complete inhibition (time 0). Then the agonist response was evaluated after washing with drug-free medium, which was

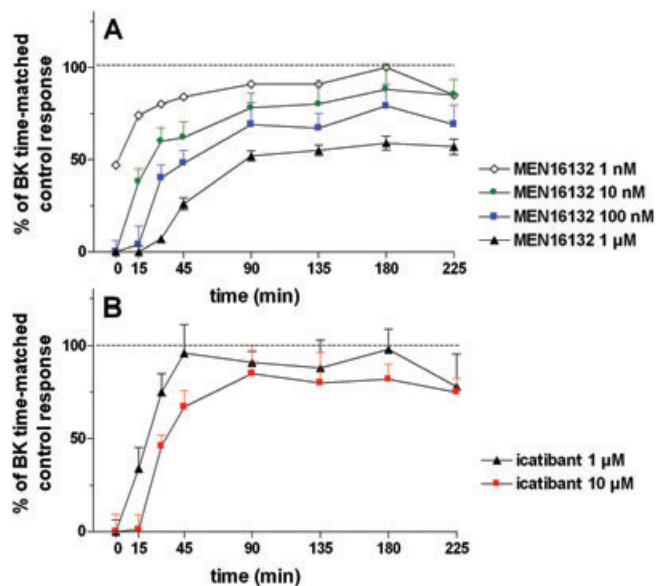


Figure 2

Reversibility of kinin B₂ receptor blockade induced by MEN16132 (A) and icatibant (B) in the inositol phosphate accumulation assay performed in CHO cells stably expressing the human recombinant B₂ receptor. Cells were incubated for 15 min with antagonists at 37°C at the indicated concentrations, and then responses to bradykinin (BK) (3 nM incubated for 60 min) were obtained, immediately or after different periods during which the medium was renewed every 15 min. Data are expressed as per cent control time-matched responses (100%), and are shown as mean ± SEM of 3–5 experiments, each one performed in triplicate.

renewed every 15 min (wash period) along a period of 3 h and 45 min (a longer time could not be used because of the significant decay of BK control response under the present experimental settings in both cell assays). The rate of recovery of BK functional response in cells pre-exposed to MEN16132 was dependent on the concentration of antagonist used (Figure 2A). Icatibant was used at two concentrations, 1 and 10 μM, which both blocked the BK-induced IP accumulation (time 0, Figure 2B), and the time-course of the functional BK response recovery was faster than that obtained with MEN16132.

As at the higher concentrations of MEN16132 used, the recovery of BK response was not complete (Figure 2A), a further set of experiments was performed to confirm that by increasing the number of washes it was possible to obtain a greater recovery of agonist functional response. Thus the effect on the reversibility of functional blockade was checked by pretreating CHO cells with 100 nM MEN16132 and comparing 15 min versus 5 min washing periods. After 90 min the BK response was 58 ± 5% and 87 ± 4% of time-matched control responses (cells which were not pretreated with the antagonist but that were exposed to the same washing protocol) respectively (data not shown).

Similar data were obtained with synovial cells (Figure 3). The inhibition exerted by icatibant was quickly reversed. At 1 μM antagonist concentration, the BK response after three washing periods (45 min) was 90 ± 3% of time-matched

control response, and at 10 μ M antagonist concentration it was 97 ± 16 after six washing periods (90 min). On the contrary, MEN16132 antagonism was reversed more slowly. After 15 washing periods (3 h and 45 min, as limit time of the experiment, see above) the BK response was $98 \pm 9\%$, $86 \pm 4\%$ and $53 \pm 5\%$ of the time-matched controls in cell pretreated with 10 nM, 100 nM and 1 μ M MEN16132 concentration.

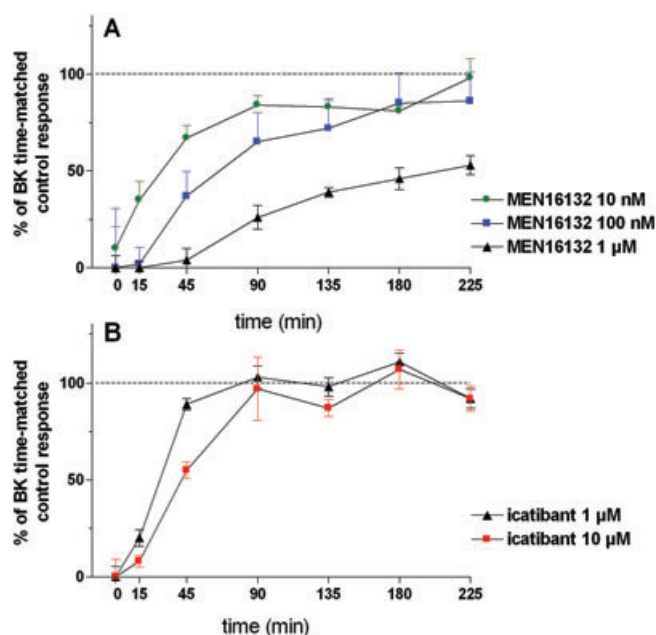


Figure 3

Reversibility of kinin B₂ receptor blockade induced by MEN16132 (A) and icatibant (B) in the inositol phosphate accumulation assay performed in human fibroblast-like synovial cells. Cells were incubated for 15 min with antagonists at 37°C as described under *Methods*, and then responses to bradykinin (BK) (3 nM incubated for 60 min) were obtained, immediately or after different periods during which the medium was renewed every 15 min. Data are expressed as per cent control time-matched responses (100%), and are shown as mean \pm SEM of 3–5 experiments, each one performed in triplicate.

Table 2

Comparison of icatibant and MEN16132 affinity at wild type and mutant human B₂ receptors (hB₂R)

hB ₂ R	TM	MEN16132 K _i [nM] (95% CI)	[³ H]-MEN11270 binding F _{mut}	icatibant K _i [nM] (95% CI)	F _{mut}
wild type		0.6 (0.4–0.8)	1	0.28 (0.17–0.50)	1
W86A	2	717 (450–1140)	1195	0.79 (0.47–1.05)	2.8
F259A	6	0.3 (0.2–0.5)	0.5	0.19 (0.15–0.32)	0.7

The affinity was evaluated at the peptide antagonist [³H]-MEN11270 radioligand binding site. F_{mut} index was calculated as K_i (mutant receptor)/K_i (wild type receptor), and corresponds to fold decrease in affinity. F_{mut} indexes greater than 3 are shown in bold type. TM indicates the transmembrane portion of the receptor where is located the mutated amino acid residue. Data are from three independent experiments, each one performed in duplicate. K_i values measured for MEN16132 were previously presented (Cucchi *et al.*, 2005).

Icatibant affinity is not affected by point-mutations which impair the affinity of MEN16132

The affinity of icatibant was determined with 17 selected point-mutated human B₂ receptors previously used to characterize the profile of MEN16132 (Cucchi *et al.*, 2005). All the selected mutations were localized in the TM segments of the B₂ receptor sequence. Inhibition curves to icatibant were performed at the radiolabelled agonist [³H]-BK (Table 1) or antagonist [³H]-MEN11270 (Table 2) binding site. This tritiated antagonist was used on those mutations which abolished the binding of BK (W86A and F259A, Meini *et al.*, 2002).

No differences in icatibant affinity could be observed at most of the mutated residues, even those which impaired the binding affinity of MEN16132 (Cucchi *et al.*, 2005). The only mutations which decreased icatibant affinity by three to five-fold were I110A (TM3), Y295F (TM7) and the double mutant W256A/Y295F (TM6/7) (Table 1).

Molecular model of the human B₂ receptor and MEN16132 complex

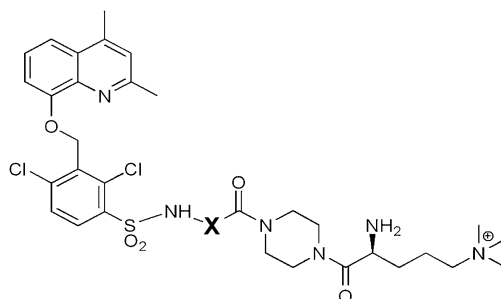
Experiments were performed to define the receptor regions corresponding to the tetrahydropyranil and the quaternary ammonium groups which were considered to favour the high antagonist potency of MEN16132.

First, the affinity of MEN16132 was compared to that of a series of derivatives differing in the α,α -disubstituted amino acid, as indicated in Table 3. These compounds were evaluated by their inhibition of the antagonist [³H]-MEN11270 binding site at the wild type and W86A mutated B₂ receptors. In this set of experiments, the affinity of MEN16132 was decreased by 1782-fold at the mutant receptor as compared with the wild type, again indicating a strong involvement of the W86 residue in the MEN16132 interaction. The rank order of F_{mut} indexes (Table 3) indicated a stronger interaction by structures which display a more extended lipophilic character and the capability to form hydrogen bond. Inhibition curves are shown in Figure 4 and highlight that the differences found at the wild type receptor (Figure 4A) are indeed flattened by the W86A mutation (Figure 4B).

In a second set of experiments the antagonist potency of MEN16132 was tested in the IP accumulation assay

Table 3

Comparison of affinity of MEN16132 and a series of derivatives substituted in the place of the tetrahydropyranyl group (X) at the wild type and W86A mutant human B₂ receptor



Compound	i.d. compound ¹	X	K _i wild type (nM)	K _i W86A mutant (nM)	F _{mut}
MEN16132	72		0.4 (0.3–0.6)	713 (340–1500)	1782
MEN15880	63		1 (0.7–1.3)	650 (330–1320)	650
MEN16318	70		12 (8–20)	2860 (750–5000)	238
MEN16298	68		3.4 (1.8–6.3)	808 (330–1975)	237
MEN16303	69		13 (11–16)	525 (200–2400)	40

F_{mut} index was calculated as K_i (mutant receptor)/K_i (wild type receptor), and corresponds to fold decrease in affinity.

¹Compound i.d. from Fattori *et al.* (2007).

performed at the wild type in comparison with the double mutant D266A/D284A B₂ receptor. Since this mutation abolished the binding of the peptide agonist (BK) and peptide antagonists (both MEN11270 and icatibant) (Bellucci *et al.*, 2004), the antagonist potency was evaluated towards the functional responses induced by the non-peptide B₂ receptor agonist FR190997, whose pharmacological profile is not affected by this mutation. In the present experiments, FR190997 induced IP accumulation with similar potency at the wild type and D266A/D284A B₂ receptor, EC₅₀ values being 0.94 nM (0.82–1.07, 95% c.l.) and 0.57 (0.48–0.68, 95% c.l.) respectively. MEN16132 antagonist behaviour was evaluated towards the agonist concentration–response curve (Figure 5), and at 1 nM concentration it shifted the agonist

curve obtained in the cells expressing the wild type receptor by 4.7-fold, and by 2.1-fold in the D266A/D284A mutant, which yielded pA₂ values of 9.57 and 9.04 respectively. At 10 nM antagonist concentration, the calculated pA₂ values were 9.42 and 9.10 in the cells expressing the wild type receptor and the D266A/D284A mutant B₂ receptor respectively. This difference was minor when MEN16132 was tested at 100 nM concentration, such as the calculated pA₂ values were 9.03 in the cells expressing the wild type and 8.88 in those expressing the mutant B₂ receptor. On the contrary, with this latter antagonist concentration a significant difference was evident when comparing the maximal agonist effect obtained in the presence of MEN16132 in the cells expressing the wild type (62 ± 3%) with that obtained in the cells

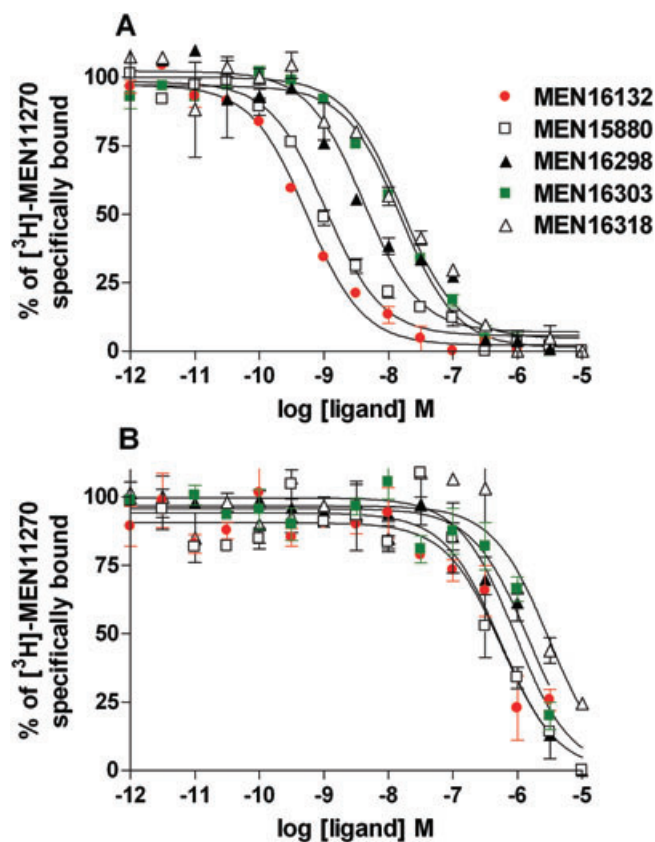


Figure 4

Competition binding curves of MEN16132 and a series of its derivatives at the peptide antagonist ([³H]-MEN11270) specifically bound to the wild type (A) and W86A mutant (B) human B₂ receptor. Compound structures and their affinity values are reported in Table 3.

expressing the D266A/D284A mutant B₂ receptor ($80 \pm 2\%$), which indicated a greater insurmountable antagonist effect at the wild type than at the mutant receptor.

Discussion

The present pharmacological analysis shows that the different pharmacodynamic properties displayed by the two kinin B₂ receptor antagonists MEN16132 and icatibant are based on a different reversibility, that is, rate of loss from the receptor compartment where they exert the functional blockade of BK responses, and on their different recognition sites in the human B₂ receptor.

Previous studies performed at the recombinant (CHO cells) and native (human lung fibroblasts, HLF-1) human B₂ receptor in membrane preparation assays indicated similar binding affinity values towards the radiolabelled agonist binding site for MEN16132 and icatibant (pK_i values in the range 10.1–10.6; Cucchi *et al.*, 2005). On the other hand, MEN16132 was about 60-fold more potent than icatibant in antagonizing BK-mediated IP stimulation both in CHO cells expressing the human B₂ receptor (pK_B values 10.3 and 8.5 for

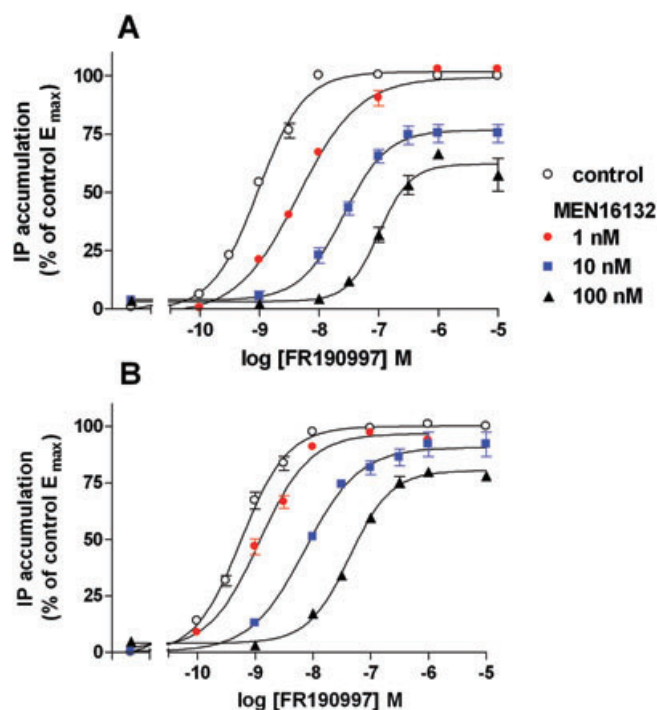


Figure 5

MEN16132 antagonism of inositol phosphate (IP) accumulation induced by the B₂ receptor non-peptide agonist FR190997 at the wild type (A) and double mutated D266A/D284A (B) human B₂ receptor expressed in CHO cells.

MEN16132 and icatibant respectively; Bellucci *et al.*, 2004; Cucchi *et al.*, 2005) and in human fibroblast-like synoviocytes (pK_B values 9.9 and 8.1 for MEN16132 and icatibant respectively; Bellucci *et al.*, 2009). Thus, we first checked the similar affinity of MEN16132 and icatibant at the BK binding site in membranes of both CHO and synovial fibroblasts, under the same experimental settings, as previous data obtained with the latter cell model were obtained in cultured cells (pK_i 8.9 and 8.4 for MEN16132 and icatibant respectively; Bellucci *et al.*, 2009). Then the kinetics of the B₂ receptor blockade exerted by the two antagonists was estimated by using a functional experimental approach. The time-course of reversibility of B₂ receptor blockade was evaluated through washout experiments in the IP accumulation assay in both CHO cells and human synovial cells. Data presented here indicate that MEN16132 dissociates from the receptor compartment more slowly than icatibant, when the two antagonists are evaluated at the same concentration. Alternatively, it can be conceived that a concentration of icatibant two orders of magnitude greater than that of MEN16132 is necessary to obtain the same time-course of functional recovery from B₂ receptor blockade, and this supports their difference in antagonist potency (estimated as pK_B values, as reported above). Present findings agree with those previously obtained in a different assay (rat uterus contractility, Meini *et al.*, 2009).

In the second part of this investigation the characterization of the two B₂ receptor antagonists at 17 mutant receptors is presented, in order to confirm that the two compounds

interact at different levels in the receptor membrane portion. Amongst the mutant receptors investigated, only the I110A, Y295F and W256A/Y295F mutations modestly decreased the affinity of icatibant. Contrary to what observed at the rat and human kinin B₂ receptor by Jarnagin *et al.* (1996) and Marie *et al.* (2001) who investigated the binding profile of icatibant by using the radiolabelled antagonist, we found a decrease in affinity of this antagonist only with the Y295F mutant (but not with the Y295A). Different reasons may be responsible for this divergent results, including the fact that, in this study, the affinity is measured at the radiolabelled agonist binding site, and the fact that these mutations *per se* affect the recognition site of BK, so that both affinity or efficacy are altered (Bellucci *et al.*, 2003). Indeed it is difficult to attribute meaningful causes at the modest reductions in affinity observed for icatibant from presented data (three to fivefold), whilst it can be inferred that the binding interaction of this antagonist with the kinin B₂ receptor is not hampered by any of the mutations which indeed decreased the affinity of MEN16132 (Cucchi *et al.*, 2005), and that the two antagonists recognize the receptor protein at different levels.

The last part of this investigation aimed at producing a model of MEN16132 docked to the human B₂ receptor. Using mutagenesis of the human B₂ receptor, we previously presented data on the essential role of the quinoline moiety of non-peptide antagonists and its interaction with some residues belonging to TM3, TM6 and TM7 (Meini *et al.*, 2004), which together define a lipophilic receptor binding crevice in a part of the receptor involved in the balance of active/inactive protein conformers, either for the human B₂ receptor or other GPCRs (Gether and Kobilka, 1998; Marie *et al.*, 2001; Rosenbaum *et al.*, 2009). In this study, our efforts are focused on finding specific receptor counterparts for the other pharmacophores of MEN16132, such as the tetrahydropyranyl and the quaternary ammonium groups, which were known to be important for high affinity ligand–receptor interaction from structure–activity relationship studies (Fattori *et al.*, 2006; 2007). The W86 residue was identified as being involved both in the binding interaction of agonist and other non-peptide antagonists besides MEN16132 (Meini *et al.*, 2002; Bellucci *et al.*, 2003), but not peptide antagonists such as icatibant or MEN11270 (this study; Meini *et al.*, 2002). The hypothesis that this functional group lies close to the W86 residue in the upper portion of TM2, which is involved in agonist receptor activation, can account for the potent antagonist properties of MEN16132. A series of derivatives bearing different groups in the place of the tetrahydropyranyl group (present in the MEN16132 structure) were assessed in displacing the radiolabelled peptide antagonist MEN11270 bound to the wild type and to the W86A mutant. The results obtained at the wild type receptor indicate (Figure 4 and Table 3) that indeed the ligand–receptor interaction is favoured (higher affinity) by the compound bearing the tetrahydropyranyl group (MEN16132) which can have a lipophilic interaction with the aromatic ring of W86. This interaction can also be stabilized by a hydrogen bond interaction between the oxygen of the tetrahydropyranyl group and the basic nitrogen of the W86 receptor residue (Figure 6). This high affinity interaction is then progressively diminished by groups which maintain lipophilic properties but do not have the capability to form a hydrogen bond (cyclopent-

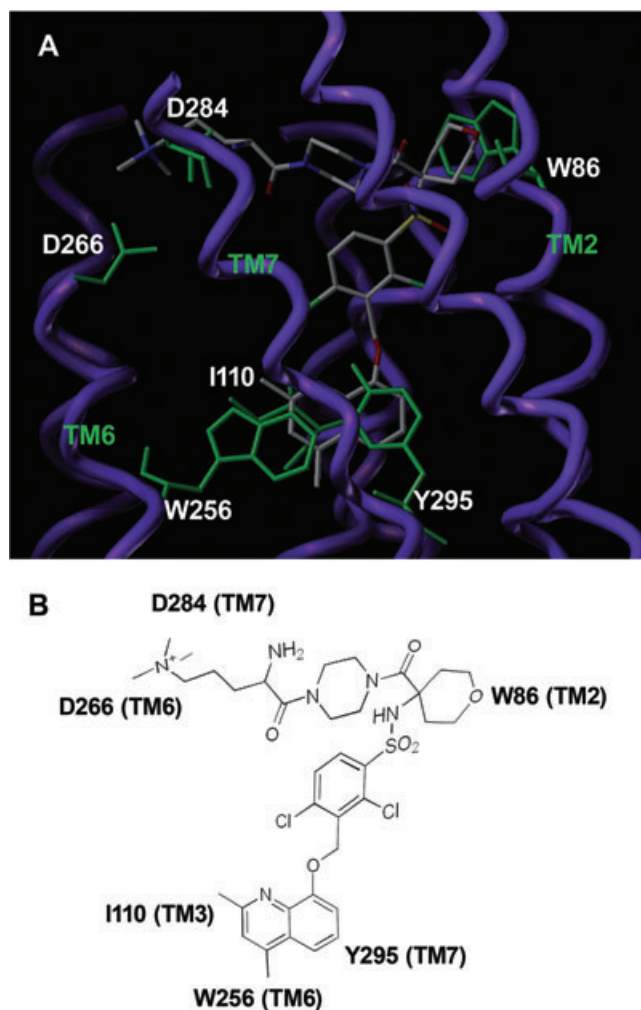


Figure 6

Model of the B₂ receptor antagonist MEN16132 docked at the human B₂ receptor, as viewed from the transmembrane side (A) and schematic representation of the putative ligand–receptor interactions (B). The quinoline group lies in a binding pocket comprised between I110 (TM3), W256 (TM6) and Y295 (TM7), and with this latter residue forms both a hydrogen bond (between the nitrogen of the quinoline group and the hydroxyl function of Tyr residue) and an aromatic–aromatic interaction. The tetrahydropyranyl ring engages with W86 (TM2) through lipophilic (aromatic–aromatic) and hydrogen bond (between the oxygen of the tetrahydropyranyl group and the nitrogen of Trp residue) interactions, whereas the positively charged quaternary ammonium of MEN16132 forms a salt bridge with the two aspartic acid residues, D266 (at the top of TM6) and D284 (top of TM7).

tyl and tetrahydrothiopyranyl groups, i.e. MEN15880 and MEN16298 respectively) or groups that bear protonated or basic amines (N-methylpiperidyl and piperidyl groups, i.e. MEN16318 and MEN16303 respectively). The fact that these observed differences among the different derivatives are not any longer evident in the W86A mutant suggested to us that the substituted group (tetrahydropyranyl group) was involved in the interaction with the tryptophan residue in TM2 (W86).

The two aspartic acid residues located at the top of TM6 and TM7, D266 and D284, respectively, appear to be in close proximity and may serve as receptor counterpart for an electrostatic interaction with a basic function. Indeed an interaction between these receptor residues and the basic arginine located in the N-terminal end of peptide ligands, both agonist and antagonists, had already been indicated both at the human and the rat B₂ receptor (Novotny *et al.*, 1994; Kyle, 1995; Jarnagin *et al.*, 1996; Bellucci *et al.*, 2004). Using the IP accumulation assay and the B₂ receptor activation induced by the non-peptide agonist FR190997, we have already demonstrated the strong interaction of icatibant with the D266 and D284 residues (Bellucci *et al.*, 2004), which displayed a pA₂ value of 8.2 at the wild type and 6.5 at the D266A/D284A mutant receptor. Present data obtained from the wild type receptor cell system indicate that MEN16132 displays an even higher antagonist potency also towards the B₂ receptor activation mediated by the non-peptide agonist FR190997 (pA₂ 9.1–9.6). We consider the reduction of potency and the minor insurmountable antagonist behaviour observed with MEN16132 at the D266A/D284A B₂ receptor mutant, as indicative of an interaction of these residues with the basic quaternary ammonium group, previously shown to contribute to the high antagonist potency of this compound (Fattori *et al.*, 2006; 2007) (Figure 6). The difference observed at the mutant D266A/D284A receptor is not as great as that previously observed with icatibant (see above), and we consider that this is due to the buried interaction of MEN16132 within the TM receptor region provided by TM3, 6 and 7 (see above) and to the fact that FR190997 interacts with some residues (i.e. I110, Y295) which are also part of the MEN16132 recognition site (Bellucci *et al.*, 2003).

As a whole, the present investigation indicates that MEN16132 has a slower reversibility than icatibant from the B₂ receptor compartment, and supports the concept that the residence time of the drug molecule on its molecular target may contribute to its long-lasting efficacy *in vivo* (Copeland *et al.*, 2006; Vauquelin and Van Liefde, 2006), and in particular for the longer duration of action of MEN16132 versus icatibant previously shown in *in vivo* animal models (Valenti *et al.*, 2008; Cialdai *et al.*, 2009). Moreover, our present findings represent an example of pharmacological assays supporting the molecular modelling, and indicate that the MEN16132 recognition site is located in the TM portion of the human kinin B₂ receptor, and involves residues contributing to receptor activation.

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Conflict of interest

None.

References

- Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). *Br J Pharmacol* 158: S1–S254.
- Aramori I, Zenkoff J, Morikawa N, Asano M, Hatori C, Sawai H *et al.* (1997). Nonpeptide mimic of bradykinin with long-acting properties at the bradykinin B₂ receptor. *Molec Pharmacol* 52: 16–20.
- Bellucci F, Meini S, Cucchi P, Catalani C, Reichert W, Zappitelli S *et al.* (2003). A different molecular interaction of bradykinin and the synthetic agonist FR190997 with the human B₂ receptor: evidence from mutational analysis. *Br J Pharmacol* 140: 500–506.
- Bellucci F, Meini S, Cucchi P, Catalani C, Giuliani S, Zappitelli S *et al.* (2004). The N-terminal of icatibant and bradykinin interact with the same Asp residues in the human B₂ receptor. *Eur J Pharmacol* 491: 121–125.
- Bellucci F, Cucchi P, Catalani C, Giuliani S, Meini S, Maggi CA (2009). Novel effects mediated by bradykinin and pharmacological characterization of B₂ receptor antagonism in human synovial fibroblasts. *Br J Pharmacol* 158: 1996–2004.
- Bond AP, Breipohl G, Worthy K, Campion G, Dieppe PA, Bhoola KD (1992). Metabolism and characterisation of kinins and Hoe 140 (kinin antagonist) in the synovial fluid of patients with inflammatory joint diseases. *Agents Actions Suppl* 38: 582–589.
- Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* 22: 3099–3108.
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS *et al.* (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318: 1258–1265.
- Cialdai C, Giuliani S, Valenti C, Tramontana M, Maggi CA (2009). Effect of intra-articular MEN16132, a kinin B₂ receptor antagonist, on nociceptive response in monosodium iodoacetate-induced experimental osteoarthritis in rats. *J Pharmacol Exp Ther* 331: 1025–1032.
- Copeland RA, Pompliano DL, Meek TD (2006). Drug-target residence time and its implications for lead optimization. *Nat Rev Drug Discov* 5: 730–739.
- Cucchi P, Meini S, Bressan A, Catalani C, Bellucci F, Santicoli P *et al.* (2005). MEN16132, a novel potent and selective nonpeptide antagonist for the human bradykinin B₂ receptor. *In vitro pharmacology and molecular characterization*. *Eur J Pharmacol* 528: 7–16.
- Dziadulewicz EK (2005). Non-peptide ligands for bradykinin receptors 1995–2004. *Expert Opin Ther Patent* 15: 829–859.
- Fattori D, Rossi C, Fincham CI, Berettoni M, Calvani F, Catrambone F *et al.* (2006). Design and synthesis of novel sulfonamide-containing bradykinin hB₂ receptor antagonists. 1. Synthesis and SAR of alpha, alpha-dimethylglycine sulfonamides. *J Med Chem* 49: 3602–3613.
- Fattori D, Rossi C, Fincham CI, Caciagli V, Catrambone F, D'Andrea P *et al.* (2007). Design and synthesis of novel sulfonamide-containing bradykinin hB₂ receptor antagonists. 2. Synthesis and structure-activity relationships of alpha, alpha-cycloalkylglycine sulfonamides. *J Med Chem* 50: 550–565.
- Fincham CI, Bressan A, Paris M, Rossi C, Fattori D (2009). Bradykinin receptor antagonists – a review of the patent literature 2005–2008. *Expert Opin Ther Pat* 19: 919–941.

- Gether U, Kobilka BK (1998). G Protein-coupled receptors. II. Mechanism of agonist activation. *J Biol Chem* 273: 17979–17982.
- Hess JF, Borkowski JA, Young GS, Strader CD, Ransom RW (1992). Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem Biophys Res Comm* 184: 260–268.
- Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G *et al.* (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro studies. *Br J Pharmacol* 102: 769–773.
- Jarnagin K, Bhakta S, Zuppan P, Yee C, Ho T, Phan T *et al.* (1996). Mutations in the B₂ bradykinin receptor reveal a different pattern of contacts for peptidic agonists and peptidic antagonists. *J Biol Chem* 271: 28277–28286.
- Kenakin TP (1997). Competitive antagonism. In: Kenakin TP (ed.). *Pharmacologic Analysis of Drug-Receptor Interaction*, 3rd edn. Lippincott Williams & Wilkins: Philadelphia, PA, pp. 331–373.
- Kyle DJ (1995). Structure-based drug design: progress toward the discovery of the elusive bradykinin receptor antagonists. *Curr Pharm Design* 1: 233–254.
- Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL (2005). International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* 57: 27–77.
- Legat FJ, Griesbacher T, Lembeck F (1994). Mediation by bradykinin of rat paw oedema induced by collagenase from *Clostridium histolyticum*. *Br J Pharmacol* 112: 453–460.
- Marie J, Richard E, Pruneau D, Paquet J-L, Siatka C, Larguier R *et al.* (2001). Control of conformational equilibria in the human B₂ bradykinin receptor. *J Biol Chem* 276: 41100–41111.
- Meini S, Quartara L, Rizzi A, Patacchini R, Cucchi P, Giolitti A *et al.* (1999). MEN11270, a novel selective constrained peptide antagonist with high affinity at the human B₂ kinin receptor. *J Pharmacol Exp Ther* 289: 1250–1256.
- Meini S, Cucchi P, Zappitelli S, Rotondaro L, Quartara L, Giolitti A *et al.* (2002). Preliminary mutational analysis of the human kinin B₂ receptor for nonpeptide antagonist ligands recognition. *Can J Physiol Pharmacol* 80: 303–309.
- Meini S, Cucchi P, Bellucci F, Catalani C, Faiella A, Rotondaro L *et al.* (2004). Site-directed mutagenesis at the human B₂ receptor and molecular modelling to define the pharmacophore of non-peptide bradykinin receptor antagonists. *Biochem Pharmacol* 67: 601–609.
- Meini S, Cucchi P, Bellucci F, Catalani C, Giuliani S, Santicioli P *et al.* (2007). Comparative antagonist pharmacology at the native mouse bradykinin B₂ receptor: radioligand binding and smooth muscle contractility studies. *Br J Pharmacol* 150: 313–320.
- Meini S, Cucchi P, Catalani C, Bellucci F, Giuliani S, Santicioli P *et al.* (2009). Pharmacological characterization of the bradykinin B₂ receptor antagonist MEN16132 in rat *in vitro* bioassays. *Eur J Pharmacol* 615: 10–16.
- Meini S, Cucchi P, Catalani C, Bellucci F, Santicioli P, Giuliani S *et al.* (2010). Radioligand binding characterization of the bradykinin B₂ receptor in the rabbit and pig ileal smooth muscle. *Eur J Pharmacol* 635: 34–39.
- Novotny EA, Bednar DL, Connolly MA, Connor JR, Stormann TM (1994). Mutation of aspartate residues in the third extracellular loop of the rat B₂ bradykinin receptor decrease affinity for bradykinin. *Biochem Biophys Res Commun* 201: 523–530.
- Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA *et al.* (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289: 739–745.
- Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC *et al.* (2007). Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450: 383–387.
- Regoli D, Barabé J (1980). Pharmacology of bradykinin and related kinins. *Pharmacol Rev* 32: 1–46.
- Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS *et al.* (2007). GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* 318: 1266–1273.
- Rosenbaum DM, Rasmussen SG, Kobilka BK (2009). The structure and function of G-protein-coupled receptors. *Nature* 459: 356–363.
- Valenti C, Cialdai C, Giuliani S, Lecci A, Tramontana M, Meini S *et al.* (2005). MEN16132, a novel potent and selective nonpeptide kinin B₂ receptor antagonist: in vivo activity on bradykinin-induced bronchoconstriction and nasal mucosa microvascular leakage in anesthetized guinea pigs. *J Pharmacol Exp Ther* 315: 616–623.
- Valenti C, Cialdai C, Giuliani S, Tramontana M, Quartara L, Maggi CA (2008). MEN16132, a kinin B₂ receptor antagonist, prevents the endogenous bradykinin effects in guinea-pig airways. *Eur J Pharmacol* 579: 350–356.
- Vauquelin G, Van Liefde I (2006). Slow antagonist dissociation and long-lasting *in vivo* receptor protection. *Trends Pharmacol Sci* 27: 355–359.
- Wirth K, Hock FJ, Albus U, Linz W, Alpermann HG, Anagnostopoulos H *et al.* (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: in vivo studies. *Br J Pharmacol* 102: 774–777.