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RESEARCH PAPER

Binding characteristics of [^{3}H]-JSM10292: a new cell membrane-permeant non-peptide bradykinin B_2 receptor antagonist

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BACKGROUND AND PURPOSE
A ^{3}H-labelled derivative of the novel small-molecule bradykinin (BK) B_2 receptor antagonist JSM10292 was used to directly study its binding properties to human and animal B_2 receptors in intact cells and to closely define its binding site.

EXPERIMENTAL APPROACH
Equilibrium binding, dissociation and competition studies with various B_2 receptor ligands and [^{3}H]-JSM10292 were performed at 4°C and 37°C. The experiments were carried out using HEK293 cells stably (over)expressing wild-type and mutant B_2 receptors of human and animal origin.

KEY RESULTS
[^{3}H]-JSM10292 bound to B_2 receptors at 4°C and at 37°C with the same high affinity. Its dissociation strongly depended on the temperature and increased when unlabelled B_2 receptor agonists or antagonists were added. [^{3}H]-JSM10292 is cell membrane-permeant and thus also bound to intracellular, active B_2 receptors, as indicated by the different 'nonspecific' binding in the presence of unlabelled JSM10292 or of membrane-impermeant BK. Equilibrium binding curves with [^{3}H]-JSM10292 and competition experiments with unlabelled JSM10292 and [^{3}H]-BK showed a different affinity profile for the wild-type B_2 receptor in different species (man, cynomolgus, rabbit, mouse, rat, dog, pig, guinea pig). Characterization of B_2 receptor mutants and species orthologues combined with homology modelling, using the CXCR4 as template, suggests that the binding site of JSM10292 is different from that of BK but overlaps with that of MEN16132, another small non-peptide B_2 receptor ligand.

CONCLUSIONS AND IMPLICATIONS
[^{3}H]-JSM10292 is a novel, cell membrane-permeant, high-affinity B_2 receptor antagonist that allows direct in detail studies of active, surface and intracellularly located wild-type and mutant B_2 receptors.

Abbreviations
AA, acetic acid; B_2 receptor, bradykinin B_2 receptor; BK, bradkyinin; JSM10292, 1-{4-methyl-3-[2-methyl-4-(4-methyl-2*H*-pyrazol-3-yl)-quinolin-8-yloxymethyl]-pyridin-2-ylmethyl}-3-trifluoromethyl-1*H*-pyridin-2-one; wt, wild-type

Introduction

The small peptides bradykinin (BK: RPPGFSPFR) and kallidin (Lys-BK) are generated through kallikreins acting on high-molecular weight precursor proteins termed kininogens. They exert their effects through the activation of the bradykinin B_2 receptor, a member of the family A of the GPCRs (Leeb-Lundberg *et al.*, 2005). Activation of the B_2 receptor is involved in a variety of (patho)physiological processes, including the regulation of blood pressure, contraction of smooth muscles, formation of oedema or transmission of pain sensation. Data obtained with B_2 receptor knockout mice suggest that kinins may also play a protective role in the prevention of diabetic nephropathy and processes accompanying aging and senescence (Kakoki *et al.*, 2006). In contrast, in 2008, the B_2 receptor antagonist icatibant was approved for the treatment of hereditary angioedema. This potentially life-threatening disease is caused by an uncontrolled release of BK, often due to the lack of C1-inhibitor and thus to the uninhibited activation of kallikrein. As icatibant is a pseudopeptide with a structure that differs from that of BK only by the presence of a few artificial amino acids it has to be applied s.c.

Most data on the binding modes of small molecule compounds are obtained indirectly through competition studies of these ligands with radiolabelled agonists or antagonists. A feasible approach for the determination of the binding sites of these small molecule compounds is to perform mutagenesis studies in combination with competition experiments. However, it is often difficult to interpret the results, when the binding sites of the radiolabelled ligands and the small molecule compounds are overlapping and, therefore, are both affected by the mutation. Moreover, competition experiments cannot identify nonspecific, receptor-independent binding, which may often be responsible for the physiological side effects of the compounds of interest.

Recently, we reported the identification of a new non-peptide, highly potent and orally available B_2 receptor antagonist, JSM10292 (Gibson *et al.*, 2009). Radiolabelling of compound JSM10292 with tritium allowed us to study directly, for the first time, the binding mode of a non-peptide B_2 receptor ligand and to determine binding characteristics that could not be revealed by indirect approaches. Thus, the purpose of the present study was to compare the binding characteristics of the non-peptide small-molecule antagonist [^{3}H]-JSM10292 with those of the peptide agonist [^{3}H]-BK by investigating various aspects such as dissociation, temperature sensitivity, or nonspecific binding. Moreover, we analysed how certain mutations in the B_2 receptor affected binding of [^{3}H]-JSM10292 and [^{3}H]-BK in comparison with another small molecule B_2 receptor ligand, MEN16132.

Methods

Gene mutagenesis and expression, cell culture

Standard PCR techniques with primers designed accordingly and the wild-type (wt) B_2 receptor gene as template were used to generate the various wt B_2 receptor mutants. In each case, successful mutation was verified by sequencing (MWG-Biotech, Ebersberg, Germany). The coding sequences of wt B_2 receptors and all mutants started with the third encoded Met (Hess *et al.*, 1992) and were cloned into the *Bam*HI and *Xho*I sites of the pcDNA5/FRT vector (Invitrogen, Groningen, The Netherlands) harbouring a haemagglutinin-tag placed between the *Hin*dIII and the *Bam*HI site. Constitutively active mutants were cloned in the pcDNA5/FRT/TO vector (Invitrogen), whereby expression in accordingly modified cells can be induced by the addition of tetracycline. For stable or inducible expression of the constructs, we used the Flp-In system from Invitrogen. In this system, the vector containing the gene of interest is inserted at a unique locus into the genome of the special host cell line Flp-In™ TREx-293 (HEK293) through the transient expression of recombinase pOG44. The HEK293 cells, cultivated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin, were transfected with HD Fugene (Roche, Mannheim, Germany) following the instructions of the manufacturer. Single stably expressing clones resulted after selection with 250 $\mu g \cdot mL^{-1}$ hygromycin B. For experiments requiring repeated rinsing of the cells, poly-D-lysine-treated (0.01% in PBS) cell culture dishes were used to ensure their adherence.

^{3}H ligand binding studies

These bindings studies were carried out as described previously (Faussner et al.*, 2005).* Confluent cells in 24 (or 48 for most experiments with [^{3}H]-BK) wells were washed three times with ice-cold PBS and incubated for the indicated time, the indicated temperature, with the indicated concentrations of ^{3}H ligand in 0.2 mL (or 0.1 mL) incubation buffer (40 mM PIPES, 109 mM NaCl, 5 mM KCl, 0.1% glucose, 0.05% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) supplemented with degradation inhibitors (2 mM bacitracin, 0.8 mM 1.10-phenanthroline and 100 μM captopril). Thereafter, cells were rinsed three times with ice-cold PBS, and the remaining radioactivity was determined after lysis of the cells with 0.2 mL of 0.3 M NaOH and transfer of the lysate to a scintillation vial. The radioactivity was measured in a β-counter after addition of scintillation fluid. Nonspecific binding was obtained in the presence of unlabelled BK or JSM10292 (at least 500-fold excess with regard to the concentration of ^{3}H ligand) and subtracted from binding in the presence of ^{3}H ligand alone to obtain specific binding.

pK_i determination

For the determination of pK_i values of various B_2 receptor ligands with 10 nM [^{3}H]-JSM10292, receptor sequestration at 37°C was inhibited by pretreatment of the cell monolayers with 100 μM phenylarsine oxide in incubation buffer for 5 min at 37°C, as described previously (Faussner *et al.*, 2004). Cell monolayers of HEK293 cells stably overexpressing the wt B_2 receptor were washed three times with PBS and incubated with stepwise dilutions of BK, icatibant, B9430 or JSM10292 in incubation buffer containing degradation inhibitors and 10 nM [^{3}H]-JSM10292. Incubation times were 30 min at 37°C or 90 min on ice. Thereafter, the remaining [^{3}H]-JSM10292 binding was determined as describe above.

[^{3}H]-JSM10292 dissociation

Cells overexpressing wt B_2 receptors on 24-well plates were rinsed with PBS and incubated with 0.2 mL of 4 nM

[^{3}H]-JSM10292 in incubation buffer with degradation inhibitors for 90 min on ice in order to obtain equilibrium binding. Thereafter, cells were washed two times with PBS to remove unbound [^{3}H]-JSM10292 and were incubated with 0.2 mL pre-warmed incubation buffer without or with 1 μM of the respective unlabelled ligand. At the times indicated, the dissociation was stopped by putting the cells on ice. The cells were washed with ice-cold PBS, and the remaining bound [^{3}H]-JSM10292 was determined after lysing of the cells with 0.3 M NaOH as described above.

[^{3}H]-JSM10292 internalization

Cells on 24-well plates were rinsed three times with PBS and incubated with 0.2 mL of 4–10 nM [^{3}H]-JSM10292 in incubation buffer with degradation inhibitors for 90 min on ice in order to obtain equilibrium binding. [^{3}H]-JSM10292 internalization was started by placing the plates in a water bath at 37°C. The internalization process was stopped at the indicated times by putting the plates back on ice and washing the cells three times with ice-cold PBS. Subsequently, surface-bound [^{3}H]-JSM10292 was dissociated by incubating the cell monolayers for 10 min with 0.2 mL of ice-cold 0.5 M NaCl/0.2 M acetic acid, pH 2.7 (NaCl/AA). The remaining monolayer with intracellular [^{3}H]-JSM10292 was lysed in 0.2 mL of 0.3 M NaOH and transferred with another 0.2 mL water to a scintillation vial. The radioactivity of both samples was determined in a β-counter after the addition of scintillation fluid. Non-receptor-related [^{3}H]-JSM10292 surface and intracellular binding were determined in the presence of 10 μM unlabelled JSM10292 and subtracted from total binding to calculate the specific values.

Data analysis

All data analysis was performed using GraphPad prism for Macintosh, Version 4.0c (GraphPad Software, Inc., San Diego, CA). All experiments were performed at least three times in duplicates, and results are presented as mean ± SEM of *n* experiments, unless indicated otherwise.

Receptor modelling

The sequence of the human B_2 receptor was aligned with the sequence of the CXCR4 chemokine receptor using ClustalW (Chenna *et al.*, 2003), indicating 22% identical amino acids and 34% similar amino acids. The B_2 receptor model was obtained by using the homology modelling tool from MOE with the following settings: 10 intermediate models were built with RMS (root mean square) gradient 1 and side chain samples at a temperature of 300 K using the amber99 force field (Wang *et al.*, 2000). Out of these 10 models, the final homology model was built by applying 'protonate 3D' before refinement with an RMS gradient of 0.5. Side chain positions were refined to optimize the protein geometry, taking into account the Ramachandran plots for typical dihedral angle distributions. Models for mutant B_2 receptors were generated using the mutation tool of MOE. Docking studies of ligands in the resulting (mutant) homology models was performed using CCDCs software GOLD (Jones *et al.*, 1995; 1997; Verdonk *et al.*, 2003). The docking results and receptor interactions were analysed with the software LigandScout (Wolber and Langer, 2005; Wolber *et al.*, 2006). In all cases, the ensemble of docking poses was analysed using pharmacophores built with LigandScout. All poses showed a very similar geometry that was in accordance with both the pharmacophore analyses and the built-in scoring function of GOLD.

Materials

Flp-In™ TREx-293 (HEK293) cells were obtained from Invitrogen. [^{3}H]-JSM10292, unlabelled JSM10292 and icatibant were generous gifts from Jerini AG (Berlin, Germany). ^{3}H labelling of JSM10292 (0.22 TBq mmol^{-1}) was performed by RC TRITEC AG (Teufen, Switzerland). [2,3-Prolyl-3,4-^{3}H]-bradykinin (2.96 TBq mmol^{-1}) was obtained from PerkinElmer Life Sciences (Boston, MA). Bradykinin was purchased from Bachem (Heidelberg, Germany). B9430 was a generous gift from Drs L Gera and J Stewart (Denver, CO). Roche (Mannheim, Germany) delivered Fugene. Poly-D-lysine, captopril, 1.10-phenanthroline and bacitracin were purchased from Aldrich (Taufkirchen, Germany). Fetal calf serum, culture media, hygromycin B and penicillin/streptomycin were obtained from PAA Laboratories (Cölbe, Germany). Primers were synthesized by MWG-Biotech (Ebersberg, Germany) and delivered desalted and lyophilized.

Results

Recently, we described the development of a series of novel small molecule antagonists of the BK B_2 receptor (Gibson *et al.*, 2009). Compound JSM10292, which proved to display the best overall properties with regard to microsomal stability, potency and oral availability, was ^{3}H-labelled with a specific activity of 6 Ci mmol^{-1} (see Figure 9D for detail) for further characterization of its binding behaviour.

Binding affinities of [^{3}H]-JSM10292 and [^{3}H]-BK for B_2 receptors are comparable at 4°C but different at 37°C

In order to compare the equilibrium binding characteristics, we performed studies with HEK293 cells stably overexpressing the wt B_2 receptor (Faussner *et al.*, 2003) and applied the ligands [^{3}H]-BK or [^{3}H]-JSM10292 at 4°C. Scatchard analysis of the curves for specific binding (Figure 1A) revealed a single binding site for both ligands with K_D values of 2.81 ± 0.44 nM (n = 7) for [^{3}H]-BK and of 4.52 ± 0.33 nM (n = 6) for [^{3}H]-JSM10292 (see also Table 1). The B_{max} values per well for the experiment, shown in Figure 1A, were 610 and 670 fmol per 24 wells for [^{3}H]-BK and [^{3}H]-JSM10292, respectively. A second experiment, again with both ^{3}H ligands applied in parallel, led to comparable results (750 fmol per 24 wells with [^{3}H]-BK vs. 870 fmol per 24 wells with [^{3}H]-JSM10292). Thus, the amount of specific binding sites detected was almost identical for both ^{3}H ligands. The most striking difference between both ^{3}H-labelled compounds was presented by their nonspecific binding. Whereas that of [^{3}H]-BK was virtually negligible (representing less than 1% of total binding), nonspecific binding for [^{3}H]-JSM10292 was considerably higher accounting for up to 25% of total binding at the higher [^{3}H]-JSM10292 concentrations (Figure 1A).

At 37°C [^{3}H]-BK binds with an approximately threefold lower affinity (K_D = 8.84 ± 0.6 nM, n = 7) than at 4°C

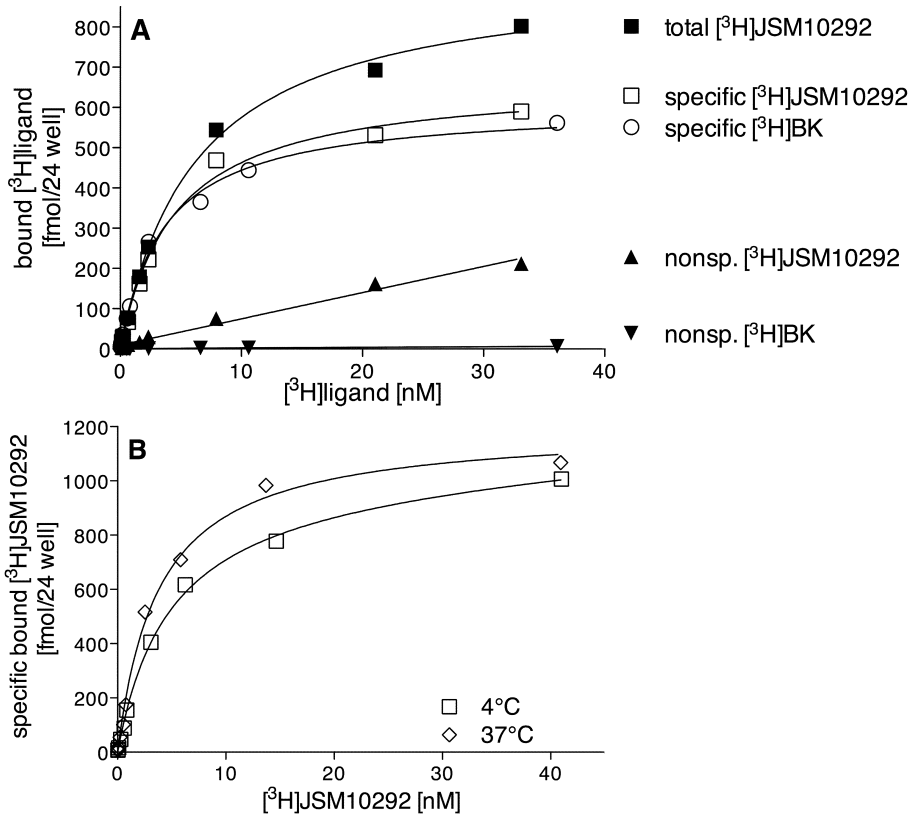

Figure 1

Saturation curves obtained (A) at 4°C with [^{3}H]-BK and [^{3}H]-JSM10292 in HEK293 cells stably overexpressing the wt B_2 receptor (total [^{3}H]-BK binding has been omitted for clarity). (B) Comparison of [^{3}H]-JSM10292 binding at 4°C and at 37°C performed in parallel. The K_D values, mean ± SEM of at least three independent experiments performed in duplicates, are given in Table 1.

Table 1

Comparison of K_D values of [^{3}H]-BK and [^{3}H]-JSM10292, and pK_i values of B_2 receptor ligands in wt B_2 receptors

	K_D (^{3}H ligand) (nM)		pK_i	
Temperature	**4°C**	**37°C**	**4°C**	**37°C**
BK	2.81 ± 0.44 (7)	8.84 ± 0.6 (7)	8.19 ± 0.14 (6)	6.85 ± 0.13 (4)
JSM10292	4.52 ± 0.33 (6)	4.05 ± 1.02 (4)	8.00 ± 0.06 (3)	8.34 ± 0.04 (3)
Icatibant			8.31 ± 0.17 (4)	7.81 ± 0.06 (4)
B9430			8.21 ± 0.21 (4)	7.77 ± 0.05 (4)

pK_i values were calculated using the corresponding K_D values for [^{3}H]-JSM10292 at 4°C and at 37°C and the IC_{50} values obtained from competition experiments performed with unlabelled compounds ($10^{-4,5}$–10^{-10} M) and 10 nM [^{3}H]-JSM10292 in HEK293 cells overexpressing wt B_2 receptors. Incubation times were 30 min at 37°C and 90 min at 4°C.

(K_D = 2.81 ± 0.44 nM, n = 7) (Table 1). In contrast, the binding affinity of [^{3}H]-JSM10292 for the wt B_2 receptor was virtually independent of the incubation temperature exhibiting K_D values of 4.05 ± 1.02 nM (n = 4) at 37°C and 4.52 ± 0.33 nM (n = 6) at 4°C (Figure 1B and Table 1). To allow the equilibrium saturation binding studies to be performed at 37°C, the cells were pretreated with the receptor internalization inhibitor phenylarsine oxide (Faussner *et al.*, 2004). There were no significant effects of the temperature or the phenylarsine oxide pretreatment on the B_{max} values with either ^{3}H ligand (not shown).

To some extent, the 'nonspecific binding' is caused by intracellularly located [^{3}H]-JSM10292

Unlabelled compounds are often used in high concentrations (up to 1000-fold the K_D) for stimulant or inhibitory purposes to ensure a high occupancy of the receptors under investiga-

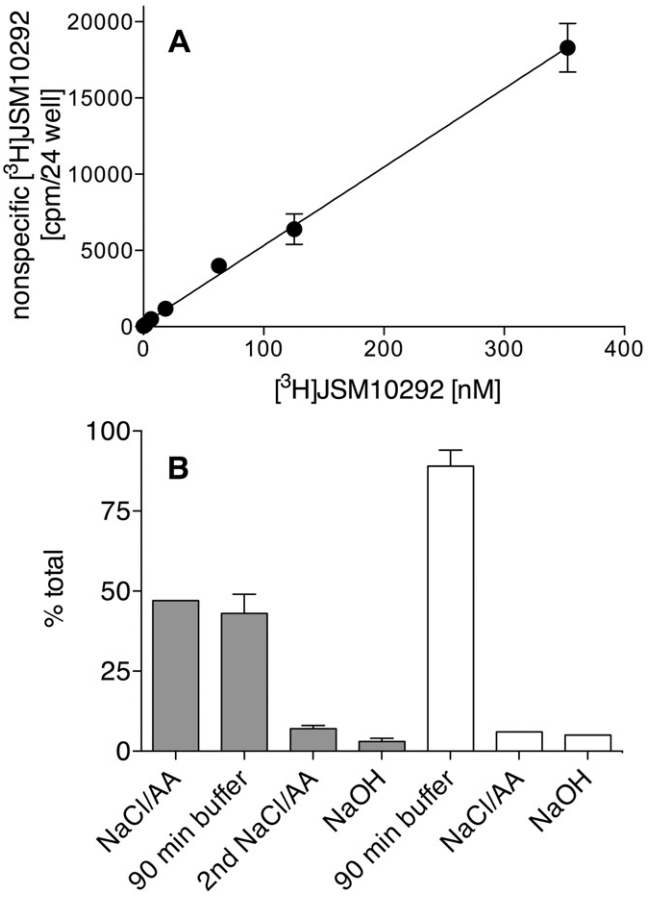

Figure 2

Nonspecific [^{3}H]-JSM10292 binding and its dissociation under different conditions. (A) Combined data for nonspecific [^{3}H]-JSM10292 obtained in saturation binding experiments on 24 wells with nine different cell lines expressing B_2 receptor mutants with low affinity for [^{3}H]-JSM10292. Their nonspecific binding was determined with an at least 500-fold excess of unlabelled JSM10292. Each point represents the mean ± SD. (B) Confluent HEK293 cells in 24 wells were pre-incubated with 200 µL of 860 nM [^{3}H]-JSM10292 at 4°C. Thereafter, cells were rinsed with PBS and incubated either first with NaCl/AA for 10 min or immediately in incubation buffer for 90 min. Subsequently, the cells were treated (again) with NaCl/AA for 10 min, and the remaining cell monolayers were lysed with 0.3 M NaOH. The columns present the percentage of the total bound radioactivity after the initial 3 h incubation (72 000 cpm per 24 wells) in each fraction (mean ± SD from determinations in triplicates). A second experiment performed with 130 nM [^{3}H]-JSM10292 (total nonspecific binding was 10 200 cpm per 24 wells) gave similar results.

tion. Nonspecific binding of compound [^{3}H]-JSM10292 was not negligible even with the relatively low concentrations used for equilibrium binding curves (see Figure 1A). Therefore, we also had a closer look at nonspecific binding at higher concentrations that reflect more closely those usually applied for experiments with unlabelled compounds. As shown in Figure 2A, the nonspecific binding is considerable – determined in the presence of an at least 500-fold excess of unlabelled JSM10292 – and shows a linear correlation to the amount of [^{3}H]-JSM10292 used in the assay. With a specific activity of [^{3}H]-JSM10292 of 6600 cpm pmol^{-1}, the amount of receptor-independently bound [^{3}H]-JSM10292 with higher ligand concentrations constituted up to several pmol [^{3}H]JSM10292 per 24 wells.

The question arose where nonspecifically bound JSM10292 is located. When naïve HEK293 cells were incubated with 860 nM [^{3}H]-JSM10292 for 3 h at 4°C and then underwent the normal wash procedure with PBS intended to remove all unbound ligand, a considerable amount of [^{3}H]-JSM10292 (approx. 11 pmol per 24 wells) was still bound to the cells (Figure 2B). A well-established procedure to remove surface bound ligand is to treat cells with 0.5 M NaCl/0.2 M acetic acid, pH.2.7 (NaCl/AA) for 10 min at 4°C (Roscher *et al.*, 1990). Such treatment, however, removed only about 50% of the non-receptor-bound ligand (Figure 2B, grey columns). Intriguingly though, despite the fact that the remaining [^{3}H]-JSM10292 could not be removed by NaCl/AA, it nevertheless dissociated almost completely when the cells were simply further incubated in incubation buffer for 90 min. This same degree of dissociation was also observed when the cells were incubated immediately in incubation buffer without prior NaCl/AA treatment (Figure 2B, open columns). The dissociation of nonspecifically bound [^{3}H]-JSM10292 was almost complete after 90 min as NaCl/AA treatment followed by lysis of the cells with 0.3 M NaOH revealed only minor amounts of [^{3}H]-JSM10292 in these fractions (Figure 2B). These results show that at 4°C with ligand concentrations usually applied for unlabelled compounds in the case of JSM10292 it is not possible to wash out the antagonist or remove it quantitatively by NaCl/AA treatment. These results suggest that a fraction of the nonspecifically bound JSM10292 is located in NACl/AA-resistant compartments inside the cells. It is not clear whether JSM10292 accumulates at specific sites or organelles within the cell. We can, however, exclude the possibility that it is trapped and enriched in acidic vesicles like lysosomes or late endosomes: pretreatment of HEK293 cells with bafilomycin or chloroquine, two compounds that prevent vesicle acidification, had no effect on the uptake (not shown) or the release of [^{3}H]-JSM10292 (Figure S1). At 37°C, the dissociation of nonspecifically bound [^{3}H]-JSM10292 proceeded much faster, and thus more than 80% of the antagonist was dissociated from the cells into the supernatant within 10 min (Figure S1).

Different affinities of B_2 receptor ligands at 4°C and at 37°C in competition binding experiments with [^{3}H]-JSM10292

The different effects of temperature on binding of [^{3}H]-JSM10292 and [^{3}H]-BK described in Figure 1A and B were also observed in competition experiments with [^{3}H]-JSM10292 in the presence of BK or two pseudo-peptides, icatibant and B9430, both characterized as B_2 receptor antagonists (Leeb-Lundberg *et al.*, 2005). At 4°C, all four unlabelled compounds displayed almost identical capacity to compete with [^{3}H]-JSM10292 for binding at the wt B_2 receptor (Figure 3A; for pK_i-values see Table 1). A clearly different picture was obtained at 37°C (Figure 3B); whereas unlabelled JSM10292 competed at 37°C and at 4°C with identical capacity, there was a strong rightward shift by more than one log unit for BK, which is in agreement with its above reported lower binding affinity at 37°C. Both pseudo-peptides also

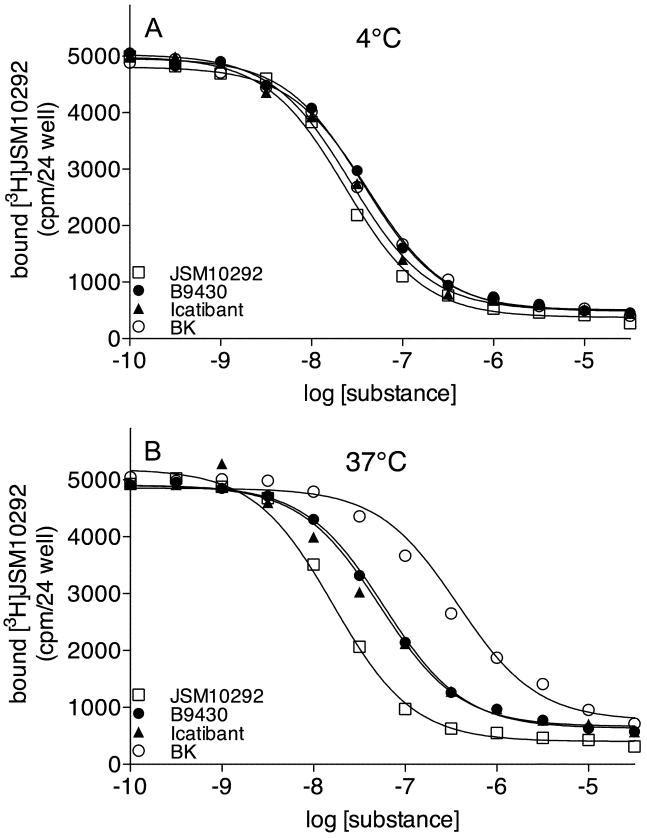

Figure 3

Competition binding curves (A) at 4°C and (B) at 37°C of 10 nM [^{3}H]-JSM10292 and increasing concentrations of different B_2 receptor ligands. The cells were pretreated with 100 μM phenylarsine oxide to prevent ligand-induced receptor internalization at 37°C. The pK_i values, mean ± SEM of at least three independent experiments performed in duplicates, are given in Table 1.

clearly displayed a shift to the right at 37°C, indicating that at 37°C they bind with a lower affinity to the wt B_2 receptors than at 4°C.

Receptor-bound [^{3}H]-JSM10292 dissociates rapidly only in the presence of other B_2 receptor ligands

Antagonists and in particular small molecule compounds often have long-lasting effects, are hard to remove by washout (Bawolak *et al.*, 2009) and may appear even insurmountable in biological assays. With unlabelled compounds, it is difficult to closely investigate the mechanisms behind these effects as their binding characteristics can be determined only indirectly via other radiolabelled ligands. Using [^{3}H]-JSM10292, it was possible to directly assess its dissociation behaviour and how it is affected by the presence of other ligands.

Once [^{3}H]-JSM10292 was bound to the wt B_2 receptor, binding at 4°C did not change for at least 30 min even in the presence of high amounts of other unlabelled B_2 receptor ligands (not shown). Even at 37°C with all unbound [^{3}H]-JSM10292 having been removed by rinsing of the cells,

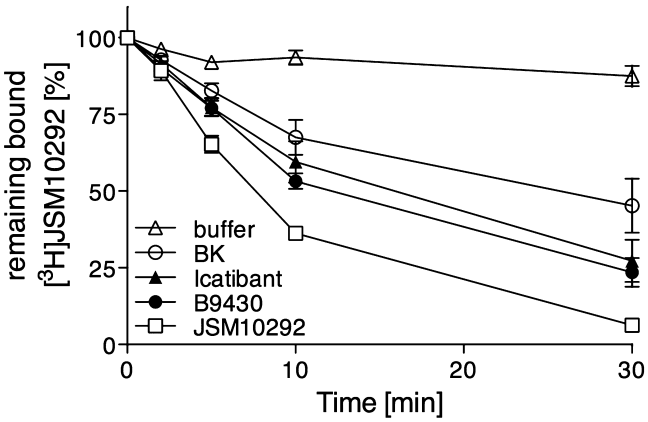

Figure 4

Dissociation curves of [^{3}H]-JSM10292 at 37°C in buffer alone or in the presence of B_2 receptor ligands. HEK293 cells overexpressing the wt B_2 receptor were incubated at 4°C for 90 min with 10 nM [^{3}H]-JSM10292. Subsequently, cells were washed at 4°C followed by incubation in binding buffer at 37°C with or without 1 μM of the indicated ligands. The remaining surface bound [^{3}H]-JSM10292 was determined at the indicated time points. Symbols represent the mean ± SEM of at least three experiments performed in duplicates.

specifically bound [^{3}H]-JSM10292 displayed little dissociation (Figure 4). Notable dissociation was only observed when other B_2 receptors ligands were present in high excess. This dissociation depended on the type of the ligand added and resulted in the following rank order: JSM10292 > icatibant = B9430 > BK, which correlates highly with the order of their potency in the competition experiments at 37°C (see Figure 3B).

[^{3}H]-JSM10292 is cell membrane-permeant and can bind to intracellular wt B_2 receptors and mutant constructs

When investigating nonspecific binding of [^{3}H]-JSM10292 at high concentrations, we had observed that a greater part of it could not be removed even by NaCl/AA treatment (see Figure 2B). Moreover, in some competition experiments, it was noticed that even high concentrations of unlabelled BK as well as the two peptidic antagonists tended not to lower [^{3}H]-JSM10292 binding to the level obtained with unlabelled JSM10292. This different nonspecific binding was observed only in cells overexpressing wt B_2 receptors and not in untransfected HEK293 cells (not shown). We hypothesized that this difference could be due to smaller numbers of intracellularly located wt B_2 receptors accessible only to the small molecule compound JSM10292 but not to the peptidic ligands. If so, specific binding of [^{3}H]-JSM10292 determined with unlabelled BK would assess only receptors on the cell surface. There, the B_2 receptor is almost exclusively located as, for example, demonstrated by GFP fusion constructs (Kalatskaya *et al.*, 2004; Bawolak *et al.*, 2009). In contrast, the use of unlabelled JSM10292 would give specific binding comprising all binding competent receptors (i.e. cell surface and intracellularly located ones). Unfortunately, the wt B_2 receptor was not very suitable for the investigation of this hypoth-

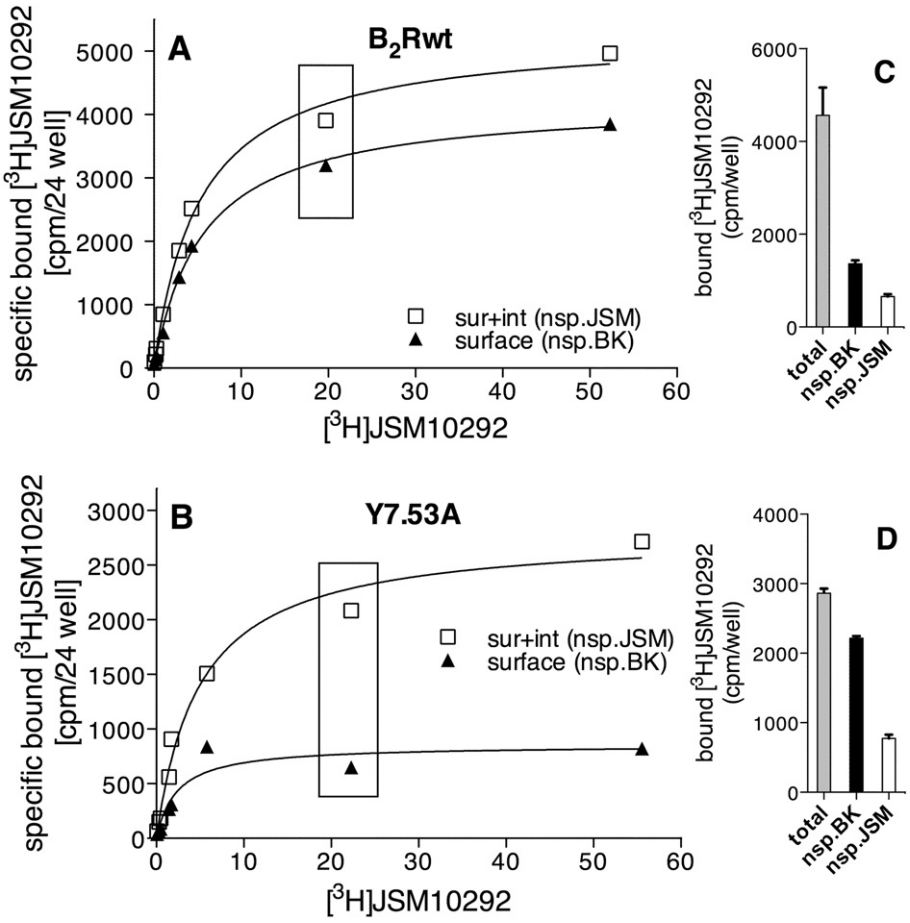

Figure 5

Saturation curves obtained after a 3 h incubation at 4°C for the assessment of surface and intracellular receptors for wt B_2 receptors (A) and mutant Y7.53A (B). The total amount of receptors (sur + int) was calculated by determination of nonspecific binding with unlabelled JSM10292. The amount of surface receptors (surface) was determined by nonspecific binding obtained with unlabelled BK. (B) and (D) Examples of total and nonspecific binding of the specific values calculated, marked in (A) and (C), respectively. Each point represents the mean from duplicate determinations. The mean ± SEM (from three independent experiments) for K_D values of total and surface receptors did not differ significantly for each of the cell lines (see text).

esis, because it is located almost exclusively at the cell surface, as demonstrated by GFP fusion constructs (Kalatskaya *et al.*, 2004; Bawolak *et al.*, 2009). However, we showed previously that mutation of a highly conserved tyrosine in the NPxxY sequence of the B_2 receptor to an alanine (termed construct Y7.53A) results in constitutive phosphorylation and consequently a strong intracellular localization of this mutant (Kalatskaya *et al.*, 2004). Thus, [^{3}H]-JSM10292 saturation binding curves were performed with HEK293 cells (over)expressing mutant Y7.53A or wt B_2 receptors. Nonspecific binding was determined either with a high excess of unlabelled JSM10292 (the calculated specific binding reflects all receptors) or BK (the calculated specific binding represents only surface receptors). As expected, with the mutant Y7.53A, we obtained strong differences in the calculated specific [^{3}H]-JSM10292 binding values (Figure 5B and D) that were not observed with the wt B_2 receptor (Figure 5A and C). Whereas for the wt B_2 receptor most of the receptors (78 ± 2%, n = 3) were located on the cell surface, only about one-third of Y7.53A mutant receptors were found extracellularly (36 ± 4%, n = 3). By comparing their apparent K_D values, we found no significant differences between surface located (4.62 ± 1.07 nM, n = 3) wt B_2 receptors versus total complement of wt B_2 receptors (4.96 ± 0.83 nM, n = 3), or between surface located (5.88 ± 1.52 nM, n = 3) Y7.53A receptors and total complement (9.86 ± 3.30 nM n = 3) of Y7.53A receptors.

[^{3}H]-JSM10292 does not induce ligand-mediated wt B_2 receptor internalization

For some GPCRs, such as the angiotensin II receptor AT_{1A} (Thomas *et al.*, 2000) or the histamine H_4 receptor (Rosethorne and Charlton, 2011), it has been shown that G-protein activation and receptor internalization can be separately addressed by ligands. Assessment of ligand-induced receptor internalization requires a technique to separate surface and intracellularly receptor bound ligand after incubation at 37°C. In the case of [^{3}H]-BK, surface receptor bound [^{3}H]-BK can be removed quantitatively by NaCl/AA treatment of the cells at 4°C. Radioactivity still associated with the cell

monolayer lysed in 0.3 M NaOH represents internalized [^{3}H]-BK (Roscher *et al.*, 1990).

Firstly, we verified that the method using NaCl/AA also works for [^{3}H]-JSM10292: on the one hand, we determined total (=surface + internal) and surface receptor bound [^{3}H]-JSM10292 using differential nonspecific binding with unlabelled BK or JSM10292 (as in Figure 5) and calculated internal [^{3}H]-JSM10292 (=total – surface [^{3}H]JSM10292). On the other hand, we treated the cells with NaCl/AA, lysed the remaining cell monolayer with NaOH and determined the amount of specific [^{3}H]-JSM10292 in both fractions.

As depicted in Figure 6A, there was a very good correlation between the data generated with either of the two methods. This demonstrates that the NaCl/AA treatment works also for [^{3}H]-JSM10292; that is, it removes only the surface bound but not the intracellularly bound [^{3}H]-JSM10292. Thus, we could use this method to determine whether incubation of the wt B_2 receptor at 37°C with [^{3}H]-JSM10292 would over time result in a reduction in NaCl/AA-dissociable [^{3}H]-JSM10292, which would suggest sequestration of the receptor. However, no reduction in surface bound [^{3}H]-JSM10292 and no increase in intracellular [^{3}H]-JSM10292 was observed during an incubation of up to 30 min at 37°C (Figure 6B). This demonstrates that JSM10292 also acts as an antagonist of ligand-mediated receptor internalization.

Binding site of [^{3}H]-JSM10292 differs from that of [^{3}H-]BK but overlaps with that of structurally related MEN16132

Previous studies have shown that small molecule compounds containing a quinoline moiety acting as agonists or antagonists for the B_2 receptor often do not share the same binding determinants as the peptide BK or the pseudo-peptide antagonist icatibant (Meini *et al.*, 2011). In order to screen for residues that may be either part of the binding site or to clearly exclude them, we screened a variety of mutants in parallel in the [^{3}H]-BK and [^{3}H]-JSM10292 binding assays. These mutants had been reported to display either poor binding of [^{3}H]-BK (Nardone and Hogan, 1994; Faussner *et al.*, 2004) or of the small molecule antagonist MEN16132 (Bellucci *et al.*, 2009; Meini *et al.*, 2011).

Mutation of negatively charged amino acids in the extracellular loops 2 or 3 (E177R; E280R), or at their transitions to the transmembrane domains (D266R, D284R), or combinations thereof (E177R/E280R, E177R/D284R), strongly affected binding of [^{3}H]-BK but not that of [^{3}H]-JSM10292 (Figure 7). On the other hand, binding of [^{3}H]-JSM10292 was strongly reduced in the double mutant V106A/I110A and the point mutant Y295F, whereas [^{3}H]-BK binding was not. Other mutations showed lower binding for both ligands indicating either that these mutants were less well expressed or that these mutations affected the binding of both ligands. However, even there differences could be seen: the mutations Y295A and S111K affected [^{3}H]-JSM10292 binding distinctly more than that of [^{3}H]-BK.

To define more closely the binding determinants of JSM10292, we measured the equilibrium dissociation constants K_D of some of these constructs and that of other mutants, which have been shown to participate in the

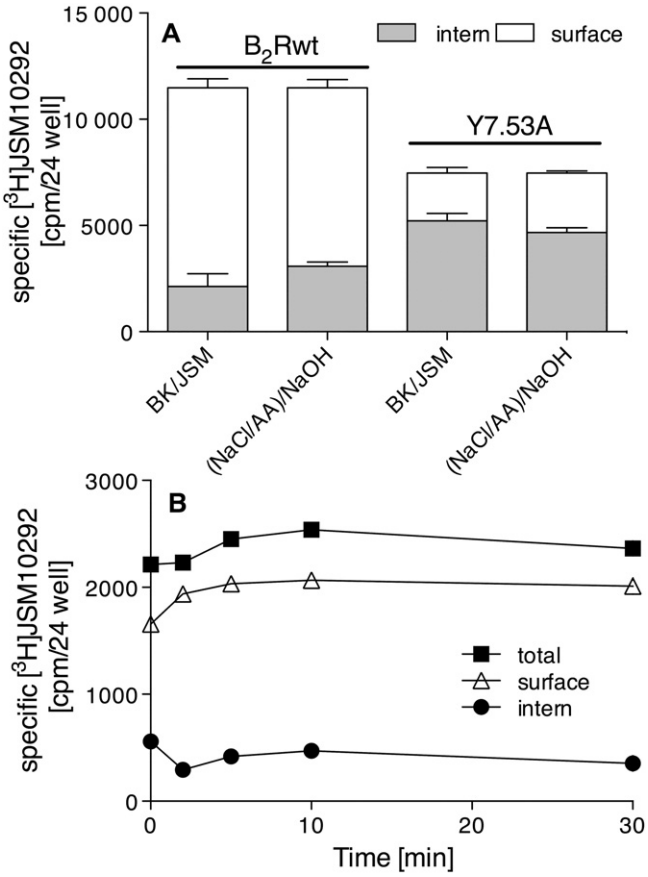

Figure 6

Comparison of the ratio of surface to total receptors determined by either differential nonspecific binding (with unlabelled JSM10292 or BK) or obtained using the NaCl/AA method. HEK293 cells (over)expressing wt B_2 receptors (B_2Rwt) or mutant Y7.53A were incubated with 30 nM [^{3}H]-JSM10292 at 4°C ± 30 μM JSM10292 or BK. Thereafter, surface and intracellularly bound [^{3}H]-JSM10292 were determined as described in the Methods section. Alternatively, surface and intracellularly bound [^{3}H]-JSM10292 were measured using NaCl/acetic acid followed by lysis of the remaining cell monolayer with 0.3 M NaOH, as described in the Methods section. (B) Lack of internalization of [^{3}H]-JSM10292 by wt B_2 receptors. Cells overexpressing wt B_2 receptors were pre-incubated with 5 nM [^{3}H]-JSM10292 on ice. Internalization was started by warming the plates to 37°C. At the indicated times, surface bound and intracellular [^{3}H]-JSM10292 were separated by NaCl/AA treatment. One representative experiment out of four is shown.

binding of small molecule compounds to the B_2 receptor or to other GPCRs of family A (Meini *et al.*, 2011).

The data in Table 2 show that only a few of the residues investigated play an important role in the binding of JSM10292. Several of them, in particular W86, I110, Y295, have also been reported to be pivotal in the binding of other B_2 receptor small molecule compounds that harbour a quinoline moiety, for example, the compound MEN16132 (Meini *et al.*, 2011) or the non-peptide agonist FR190997 (Bellucci *et al.*, 2003). Strong differences, however, can be seen with regard to the residues F259 and S111 that are crucial for binding of JSM10292 but not for that of MEN16132 (Meini

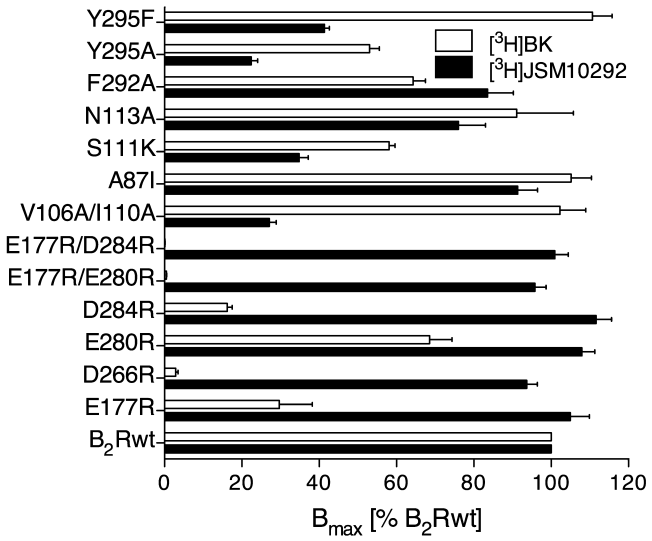

Figure 7

Comparison of [^{3}H]-JSM10292 and [^{3}H]BK binding at 4°C to wt B_2 receptors (B_2Rwt) and various mutant constructs. Specific binding to confluent monolayers of the indicated constructs on 24 wells was determined with 10 nM of the respective ^{3}H ligand and incubation for 90 min at 4°C. Binding was normalized to the respective binding to the wt B_2 receptors. The columns represent the mean ± SEM from at least three experiments performed in duplicates.

et al., 2011). These data indicate that although the binding sites of these two compounds share some residues, they are not identical.

Binding affinities of JSM10292 for wt B_2 receptor orthologues from different species vary considerably

For the future evaluation of JSM10292 in animal models, the wt B_2 receptor genes from several species were synthesized with humanized codon usage and stably expressed in HEK293 cells. Competition experiments of unlabelled JSM10292 and BK against [^{3}H]-BK revealed that binding of JSM10292 varied strongly from species to species (Figure 8). In some species, the competition curves for BK and JSM10292 did not differ at all (man, cynomolgus, mouse) or by less than one log unit (rat, rabbit, pig). In contrast, in guinea pig and dog, JSM10292 was more than one or almost two log units, respectively, less competitive than BK. A closer look at a species alignment in the region around the residues determined to be important for binding of JSM10292 (i.e. V106/I110 and Y295) indicated certain amino acids as potential candidates responsible for the difference between man and dog or guinea pig (see Figure S3).

Swapping these residues (I110 in man with the corresponding L109 in dog; S111/L114 with the corresponding Q112/115 M in guinea pig) confirmed their importance for binding of JSM10292; these changes resulted in significant increases in the binding affinities of the mutated dog and guinea pig receptors and in a deterioration of binding affinities in the mutated human receptors by 6- to10-fold (Table 3).

Discussion and conclusion

The availability of the novel B_2 receptor antagonist JSM10292 in a ^{3}H-labelled form allowed us to determine directly, for the first time, the binding properties of a small molecule B_2 receptor ligand.

Our data demonstrated that JSM10292 binds with high affinity and reversibly to the wt B_2 receptor, as it can be displaced by B_2 receptor agonists (BK) or other antagonists (B9430, icatibant) (Figure 4). JSM10292 displayed the same high affinity for the wt B_2 receptor at 4°C and at 37°C, in contrast to BK that shows a three- to fourfold affinity reduction at 37°C (Table 1). Such a reduction is typical for an agonist and may be due to the uncoupling of the receptor from its cognate G-proteins, a change in its interaction with arrestins (Gehret *et al.*, 2010) or other intracellular proteins as suggested by the ternary model (Samama *et al.*, 1993). Thus, the lack of an affinity shift for JSM10292 supports the notion that this compound is a full B_2 receptor antagonist as it does not induce any changes in the interaction of the wt B_2 receptor with intracellular proteins that would affect its affinity. The different binding affinity of BK at 37°C also explains the reduced capacity of BK to displace [^{3}H]-JSM10292 in the dissociation assay (Figure 4) and the competition binding experiment (Figure 3B). The pseudo-peptides B9430 and icatibant also induced some rightward shift in the competition assay curve at 37°C and reduced displacement as compared with unlabelled JSM10292 in the dissociation assay. This is in good agreement with the fact that under certain conditions (in particular when the expression of wt B_2 receptors is high) these compounds show some agonistic activity (Faussner *et al.*, 2009), whereas JSM10292 does not (Figure S2).

Our data demonstrated that JSM10292 is membrane-permeant and, therefore, is also able to bind to competent binding receptors inside the cell. Although this cell membrane permeability can be assumed for many lipophilic small molecule compounds, it can be proved directly only when a cell membrane-permeant radioligand is available, which is the case for only a few GPCRs. However, for example, the non-peptide antagonists of the angiotensin II AT_{1A} receptor, [^{3}H]-LF 7–0156 and [^{3}H]-DuP753, have been reported to display differential nonspecific binding with their unlabelled forms and the peptide antagonist Sar^1-AII, which also suggests they have the ability to penetrate cells (Nouet *et al.*, 1994). The fact that the reported binding experiments were performed with rat liver membranes and not with intact cells does not contradict this assumption; we also observed a strong difference in nonspecific binding determined with BK compared to JSM10292 for [^{3}H]-JSM10292 in crude membrane preparations of cells expressing mutant Y7.53A but not in those overexpressing wt B_2 receptors (data not shown). Thus, even in crude membrane preparations, apparently not all Y7.53A receptors previously located inside the cell become accessible for the hydrophilic peptide BK. Apparently, the distinction between previous surface and intracellular receptors is also, in part, maintained in crude membrane preparations.

The studies presented here demonstrate that with higher concentrations (>100 nM) of JSM10292, there is considerable non-receptor-related binding of this compound that cannot be removed by rinsing of the cells or by NaCl/AA treatment.

Table 2

Equilibrium dissociation constants K_D at 4°C for [^{3}H]-JSM10292 and [^{3}H]-BK in wild-type and mutant B_2 receptors

Construct	TM	[^{3}H]-JSM10292 K_D (nM)	*n*	[^{3}H]-BK K_D (nM)	*n*	MEN16132 K_i (nM)
B_2Rwt		4.52 ± 0.33	6	2.81 ± 0.44	7	0.09
LL79/83	2	11.91 ± 1.73	5	–		–
W86	2	>200	5	n.d.		717
V106A/I110A	**3**	**87.15 ± 13.50**	**3**	4.11 ± 0.80	4	28 (I110A)
N107A	**3**	**20.71 ± 1.67**	**3**	9.42 ± 0.82	5	–
S111K	**3**	**67.90 ± 7.56**	**3**	**10.75 ± 0.84**	8	0.11 (S111A)
N113A	3	8.84 ± 1.27	3	1.37 ± 0.51	4	–
L114A	3	2.56 ± 0.71	4	12.90 ± 1.29	2	0.10
L114W	3	6.67 ± 1.08	5	–		–
Y115A	3	3.96 ± 1.10	5	6.50	1	–
V185A	EL2	3.53 ± 0.83	4	19.57 ± 2.45	2	–
S187I	EL2	3.45 ± 0.81	5	19.99 ± 1.28	3	–
W256C	6	6.71 ± 1.35	3	15.48	1	0.44 (W256A)
F259A	6	**>200**	4	67.38	1	0.3
T263A	6	12.69 ± 2.07	5	22.22	1	–
F292A	7	9.36 ± 1.41	5	80.1 ± 0.85	9	0.22
Y295A	7	**>200**	3	15.55 ± 1.18	6	68
Y295F	7	**80.61 ± 7.41**	4	4.15 ± 1.06	3	9.0
N297A	7	13.49/8.1	2	1.44 ± 0.35	4	–
N301D	7	7.62/9.76	2	2.26 ± 0.12	4	–
Y305A	7	9.13 ± 1.00	3	2.91 ± 0.49	6	–

K_i values for MEN16132 were taken from Meini *et al.* (2011). Values in bold were significantly different from those in wt B_2 receptors ($P < 0.05$, determined by one-way ANOVA using Dunnett's multiple comparison test). Underlined values indicate an effect on JSM10292 binding strongly different from effect on MEN16132 binding.
EL2 extracellular loop 2; n.d., not detectable; TM, transmembrane domain.

Table 3

Equilibrium dissociation constants K_D of [^{3}H]-JSM10292 for human, canine and guinea pig (GP) wild-type B_2 receptors and respective mutants

Construct	K_D (nM)	*n*
human B_2Rwt	4.52 ± 0.33	6
human I110L	30.60 ± 2.78*	4
human S111Q/L114M	49.21 ± 7.73*	4
dog B_2Rwt	165.7 ± 24.57	4
dog L110I	56.91 ± 9.43***	4
GP B_2Rwt	98.24 ± 23.84	4
GP Q111S/M114L	41.67 ± 7.66*	4

n (number of experiments); numbering for guinea pig wt B_2 receptors (B_2Rwt) follows that of the respective residues in the human B_2Rwt; comparison between mutant and respective wild-type binding constants: *$P < 0.05$, ***$P < 0.001$ (one-way ANOVA using Bonferoni's multiple comparison test).

It can, however, be strongly reduced by prolonged washout (Figure 2). Therefore, it is not possible to recover the full wt B_2 receptor binding complement with a ^{3}H-labelled ligand after incubation of the cells with high concentrations of JSM10292, even after all surface bound JSM10292 had been dissociated by NaCl/AA treatment. Intracellularly located JSM10292 must also have been removed by the additional incubation for at least 90 min at 4°C (>30 min at 37°C) in incubation buffer. Otherwise, the JSM10292 residing in NaCl/AA treatment-resistant compartments would slowly diffuse out of the cells into the supernatant and compete with the radioligand in the binding assay. Similar effects can be expected in animal studies. After application of higher amounts of JSM10292, it would take some time to regain unoccupied receptors, as there would be a pool of this compound inside the cells that could not be cleared immediately via the circulation even at 37°C. This property in combination with the slow dissociation rate even in the presence of other B_2 receptor ligands (Figure 4) might – depending of course on the exact conditions of the experiment – result in the description of JSM10292 as an insurmountable ligand.

In the past few years, it has become clear that ligands (agonists or antagonists) do not necessarily affect all the

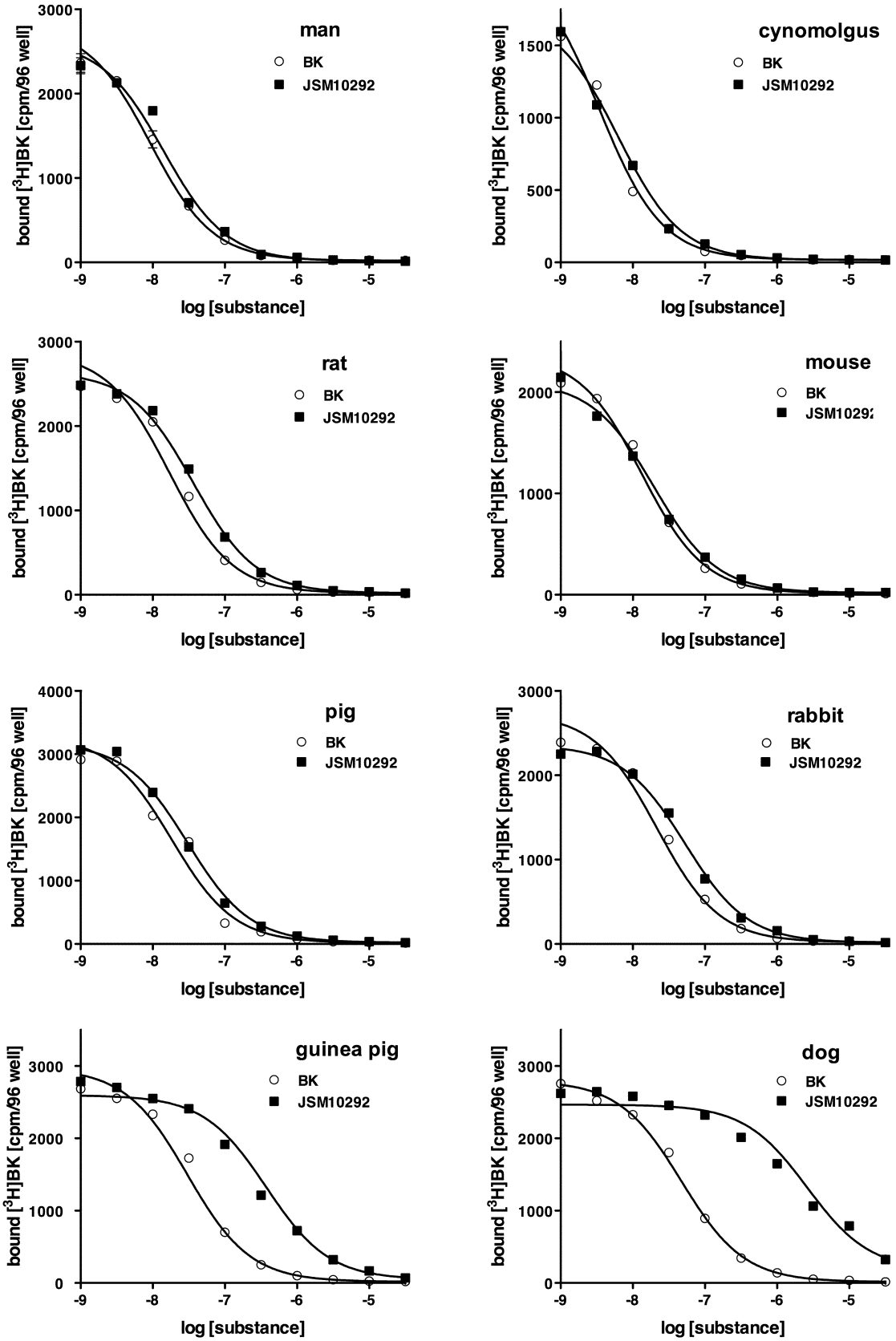

Figure 8

Competition of BK and JSM10292 against 2 nM [^{3}H]-BK at 4°C for binding at the wt B_2 receptors of different species stably expressed in HEK293 cells. One representative curve out of at least three performed in duplicates is shown for each species.

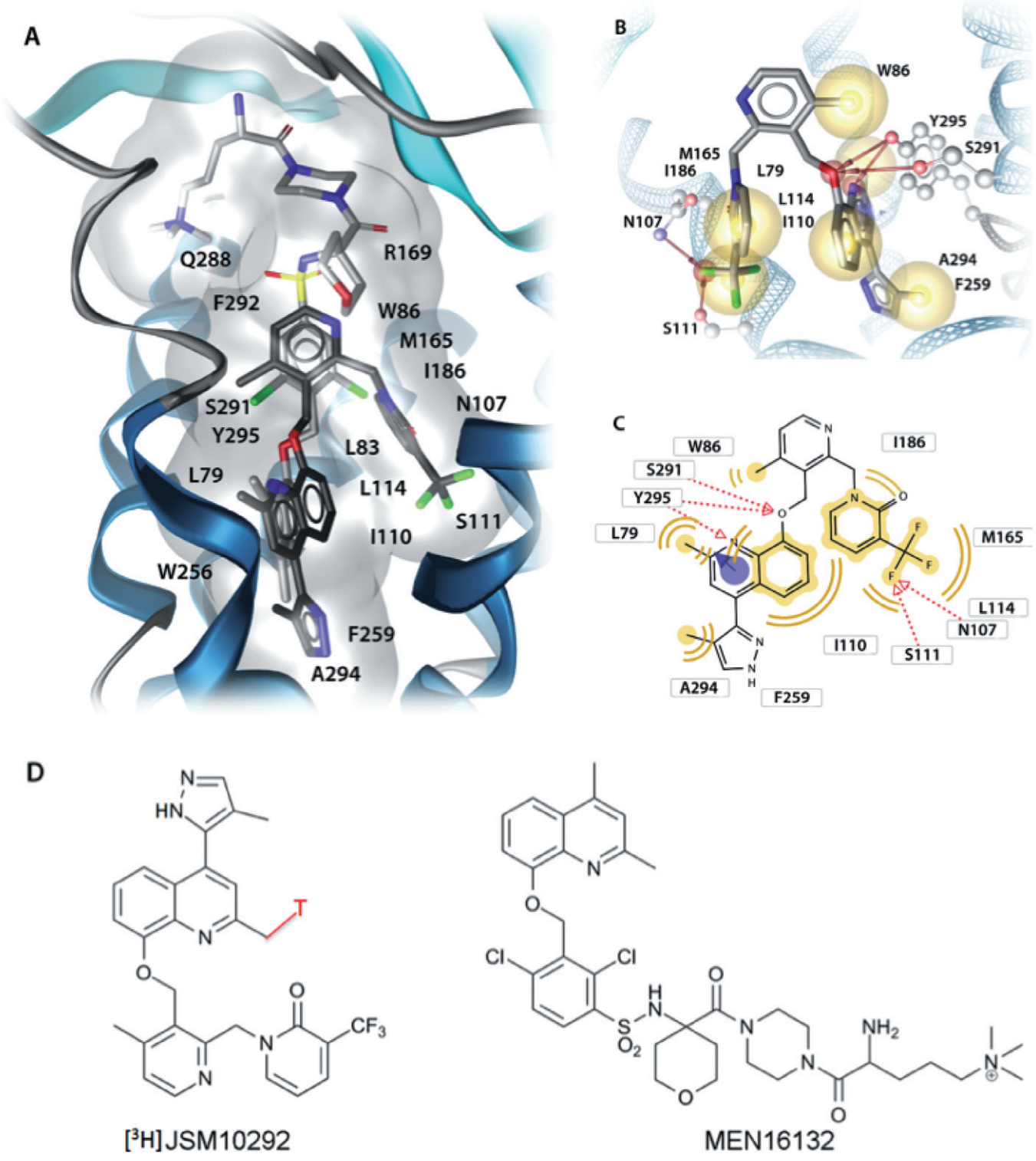

Figure 9

(A) Superimposition of JSM10292 and MEN16132 in the binding pocket. Both compounds show the same orientation of the quinoline moiety with the ether bridge to the aromatic system in both structures, while the other structural elements differ in their positions. (B) Docking of JSM10292 into the homology model of wt B_2 receptors and its interactions with the surrounding amino acids. (C) The 3D pharmacophore analysis of JSM10292 shows seven lipophilic features and five hydrogen bond acceptors, as well as a π-stacking interaction. (D) Structures of the small molecule antagonists of the B_2 receptor JSM10292 and MEN16132. The position of the tritium-label (T) in JSM10292 is shown in red.

different cellular responses that can be elicited by a respective GPCR similarly. Ligands that stimulate or inhibit these response pathways differentially were termed biased or functionally selective (Wisler *et al.*, 2007; Violin *et al.*, 2010; Butcher *et al.*, 2011; Whalen *et al.*, 2011). Our data suggest that JSM10292 is a full unbiased antagonist with regard to all the biological effects mediated by the wt B_2 receptor investigated: it does not have a lower affinity for the wt B_2 receptor at 37°C (Figures 1B and 3B) as compared with 4°C, in contrast to the full agonist BK or the pseudo-peptides B9430 and icatibant for which some partial agonistic activity has been reported (Faussner *et al.*, 2009). Moreover, JSM10292 does not elicit a response in a calcium assay (Gibson *et al.*, 2009), does not stimulate phosphoinositol hydrolysis (Figure S2) and does not induce internalization of the wt B_2 receptor (Figure 5B). However, due to its membrane-penetrating capacity it might act as a chaperone that can stabilize the conformation of intracellularly located mutants so that they can get translocated to the cell surface (Feierler *et al.*, 2011).

Meini *et al.* (2011) have recently generated a homology model of the human B_2 receptor starting from the bovine rhodopsin crystal structure and used it for docking studies with MEN16132, another quinoline-based B_2 receptor antagonist. The availability of a crystal structure for the CXCR4 chemokine receptor (PDB 3OE0), that has a higher sequence similarity to the B_2 receptor than bovine rhodopsin (19% identical amino acids vs. 22% for CXCR4, both 34% sequence similarity), has allowed us to build a new B_2 receptor homology model based on the CXCR4. The results of our docking experiments indicate that both MEN16132 and JSM10292 are located with their quinoline moiety in a lipophilic pocket formed by L79, W86, I110 and Y295, while otherwise showing only partial overlap upon binding (Figure 9A). Additionally, three hydrogen bonds are formed for both: two between the oxygen of the ether bridge and the hydroxyl function of Y295 and the hydroxyl function of S291 and a third hydrogen bond between the hydroxyl function of Y295 and the nitrogen of the quinoline moiety. Y295 interacts through π-stacking with the quinoline ring.

Our model suggests that W86 plays an essential role in the binding mode of JSM10292, because it is directly opposite the methylpyridine moiety forming a small lipophilic pocket. The trifluoromethyl group is embedded into the lipophilic region formed by amino acids I110, I186, L114 and M165 and at the same time allows for electrostatic interaction with the hydroxyl function of S111 and the amino function of N107. F259, A294 and L114 form another lipophilic area opposite the methylpyrazol ring. In contrast to MEN16132, where W256 is crucial for ligand binding (Meini *et al.*, 2002; 2004; 2011), W256 is not important for JSM10292 binding. This can be explained by the different orientation of JSM10292 and the additional stabilizing interactions of the methylpyrazol ring with F259 and A294 (Figure 9B and C).

The mutational studies support our postulated binding mode. The reduction in binding affinity accompanying the replacement of bulkier residues by alanines (V106A/I110A, F259A, Y395A) can be explained as an enlargement of the lipophilic pocket hosting JSM10292 that decreases the possibility of hydrophobic contact. In mutant Y295F, the hydrogen bond to the ether bridge is lost, which may explain the lower binding affinity. The strongly reduced affinity resulting from the S111K mutation gives support to our assumption of an electrostatic interaction between the hydroxyl group of S111 and a fluorine atom of the trifluoromethyl group. The almost complete loss of JSM10292 binding observed for mutant W86A can be explained by the importance of this amino acid for the shape of the binding site; the ligand exhibits a high degree of steric complementarity to the residues around W86. Supplemental Figure S4A and B show the different orientation of the pyridine ring in the binding pocket of the W86A mutant model, which also indicates a disruption of the π-stacking interaction to Y295.

The results of the structural analyses of the mutational models on the binding of JSM10292 are summarized in Table S1.

The docking model also gives an indication as to why, in contrast to the peptide BK, compound JSM10292 binds with highly different affinities to the wt B_2 receptor orthologues in the various species. It is a small molecule compound and, therefore, has fewer interaction surfaces with the receptor than BK, thus a different amino acid within the binding pocket almost always has a strong effect on binding. An increased species variance in the binding site of JSM10292 is also quite likely because it differs distinctly from that of BK and therefore was not subject to the same evolutionary pressure. This suggests that the choice of the right animal model for a small molecule compound becomes more difficult the more its binding site differs from that of the endogenous ligand.

Taken together, our present study demonstrates that the novel non-peptide compound JSM10292 is a reversible, unbiased, full antagonist for the B_2 receptor that displays considerable nonspecific binding at higher concentrations. Homology modelling based on the CXCR4 structure as a template suggests that its binding site differs from that of the endogenous agonist BK but overlaps with that of other non-peptide agonists or antagonists (e.g. MEN16132) that also contain a quinoline moiety. As JSM10292 is cell membrane-permeant, it can bind to intracellular wild-type and mutant B_2 receptors. In its ^{3}H-labelled form, it could be used to assess the cell penetrating capacity of other B_2 receptor ligands and to differentiate between surface and intracellular wild-type and mutant B_2 receptors.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) Effect of pretreatment with bafilomycin or chloroquine on the dissociation of wash-resistant [^{3}H]-JSM10292 at 4°C. Naïve HEK 293 cells, plated on 24 wells, were pretreated or not (control) with either 100 nM bafilomycin A1 (Baf) or 100 μM chloroquine (CQ) for 30 min at 37°C. Subsequently, the cells were washed with ice-cold PBS and incubated with 200 μL of 300 nM [^{3}H]JSM10292 for 3 h at 4°C. After a second wash with PBS, the cells were incubated in 500 μL incubation buffer at 4°C. At the indicated times, the supernatant was quantitatively transferred to a scintillation vial, and the [^{3}H] content was determined in a β-counter. Thereafter, [^{3}H] radioactivity still bound to the cells was determined after lysis of the cell monolayers with 0.3 M NaOH. The experiment shown is representative of two experiments performed in triplicates. Pretreatment did not significantly affect initial total binding (about 29.000 cpm for all three protocols). (B) Naïve HEK293 cells on 24 wells were incubated with 450 nM [^{3}H]JSM10292 at 37°C for 30 min. Thereafter, cells were washed four times with ice-cold PBS and incubated with 500 μL pre-warmed incubation buffer at 37°C. At the times indicated, [^{3}H] radioactivity in the supernatant and that still cell bound was determined as described above. Two experiments performed in triplicates are shown (total remaining binding was 45.000 cpm in experiment 1 and 34.000 cpm in experiment 2).

Figure S2 Basal and stimulated inositol phosphate accumulation in B2Rwt overexpressing HEK293. Cells on 12 wells were pre-incubated overnight with 0.5 μCi [^{3}H]inositol. Inositol phosphate accumulation was started at 37°C with 1 μM of the indicated compound in the presence of 50 mM LiCl and continued for 30 min. Thereafter, total inositol phosphates were determined by anion exchange chromatography (Faussner *et al.*, 2009). The results (means ± SEM from at least three independent experiments performed in duplicates) are given as *x*-fold above total inositol phosphates determined in unstimulated cells kept at 4°C.

Figure S3 Alignment of B2Rwt orthologs from different species. Residues mutated only in the human B2Rwt are highlighted brown, those swapped between human and dog B2Rwt are highlighted yellow and those between human and guinea pig B2Rwt black.

Figure S4 Close-up of the docking of JSM10292 into the homology model of B2R wild-type (A) and mutant W86A (B).

Table S1 Protein–ligand interactions of JSM10292 according to the docking model shown in Figure 9.

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