SSR240612, A NEW NON-PEPTIDE ANTAGONIST OF THE BRADYKININ B₁ RECEPTOR. BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION


Sanofi-Synthelabo Recherche, Montpellier, France
SSR240612, a new antagonist of the bradykinin $B_1$ receptor

Corresponding author

Jean GOUGAT
Sanofi-Synthelabo Recherche
371 rue du Prof. J. Blayac
Cedex 04
34184 Montpellier
France
Telephone: +33 4 67 10 6332; Fax +33 4 67 10 53 46
E-mail: jean.gougat@sanofi-synthelabo.com

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ABBREVIATIONS: CHO, Chinese hamster ovary; HEK, human embryonic kidney; BK, bradykinin; MPO, myeloperoxidase; ANOVA, analysis of variance; SSR240612, (2R)-2-\[((3R)-3-(1,3-benzodioxol-5-yl)-3-\{[(6-methoxy-2-naphtyl)sulfonyl]amino\}propanoyl) amino]-3-\{4-\{2R,6S)-2,6-dimethylpiperidinyl\}methyl\}phenyl)-N-isopropyl-N-methylpropanamide hydrochloride
ABSTRACT

The biochemical and pharmacological properties of a novel non-peptide antagonist of the Bradykinin B₁ receptor, SSR240612, were evaluated. SSR240612 inhibited the binding of [³H]Lys⁰-desArg⁹-BK to the B₁ receptor in human fibroblast MRC5 and to recombinant human B₁ receptor expressed in HEK cells with inhibition constants (Ki) of 0.48 nM and 0.73 nM, respectively. The compound selectivity for B₁ versus B₂ receptors was in the range of 500 to 1000 fold. SSR240612 inhibited Lys⁰-desAr⁹-BK (10 nM) induced inositol monophosphate formation in human fibroblast MRC5, with an IC₅₀ of 1.9 nM. It also antagonized desArg⁹-BK induced contractions of isolated rabbit aorta and mesenteric plexus of rat ileum with a pA₂ of 8.9 and 9.4, respectively. Antagonistic properties of SSR240612 were also demonstrated in vivo. SSR240612 inhibited desArg⁹-BK induced paw edema in mice (3 and 10 mg/kg p.o. and 0.3 and 1 mg/kg i.p.). Moreover, SSR240612 reduced capsaicin-induced ear edema in mice (0.3, 3 and 30 mg/kg p.o.) and tissue destruction and neutrophil accumulation in the rat intestine following splanchnic artery occlusion/reperfusion (0.3 mg/kg i.v.). The compound also inhibited thermal hyperalgesia induced by UV irradiation (1 and 3 mg/kg p.o.) and the late phase of nociceptive response to formalin in rats (10 and 30 mg/kg p.o.). Finally, SSR240612 (20 and 30 mg/kg p.o.) prevented neuropathic thermal pain induced by sciatic nerve constriction in the rat. In conclusion, SSR240612 is a new, potent, and orally active specific non-peptide bradykinin B₁ receptor antagonist.
Kinins are 9-11 amino acid peptides known to be important mediators of pain, inflammation, and cardiovascular homeostasis. They are released in injured tissues from kininogen, by activation of plasma or tissue kallikreins (Bhoola et al., 1992). Kinins exert their biological activities via the activation of two sub-types of G-protein coupled receptors, denoted B1 and B2 receptors (Regoli and Barabé, 1980; Regoli et al., 1998). Bradykinin (BK) and Lys0-BK are natural endogenous agonists of bradykinin B2 receptors, while their kininase I hydrolyzed metabolites desArg9-BK and Lys0-desArg9-BK are specific agonists of bradykinin B1 receptors. B1 peptide antagonists were obtained by replacing the C-terminal Phe residue of agonists by Leu. Human and rabbit B1 receptors have a higher affinity for Lys0-desArg9,Leu8-BK than for desArg9,Leu8-BK whereas rat and mice receptors have equivalent affinities for these two antagonists (Regoli et al. 1998; Jones et al., 1999). Whereas the B2 receptors are constitutively present in normal tissues, the B1 receptors are poorly present in healthy tissues, but highly inducible by tissue injury and treatment by bacterial endotoxin or inflammatory mediators such as cytokines (Marceau et al., 1998).

B1 receptors are important mediators of chronic inflammation, especially with an immune component (Couture et al., 2001). The contribution of B1 receptor activation in inflammation and pain process is supported by the demonstration that B1 receptor knockout mice have a decreased response to nociceptive and proinflammatory stimuli. Mice lacking B1 receptors show hypoalgesia and a decrease in neutrophil migration, in a pleurisy and a peritonitis model (Pesquero J. B. et al., 2000; Araujo et al., 2001). The hyperalgesic response to Freund’s adjuvant-induced inflammation is blunted (Ferreira et al., 2001). The therapeutic interest of B1 receptor blockage is further supported by the pharmacological properties of B1 peptide antagonists. Edema and hyperalgesia associated to burn injuries are attenuated by desArg9,Leu8-BK (Perkins et al., 1993; Rawlingson et al. 2001). The peptide inhibitor R-954 was shown to reduce eosinophil and neutrophil infiltration and airway hyper reactivity.
induced by lung inflammation (Gama Landgraf et al., 2003). DesArg⁹,Leu⁸-BK also inhibits carrageenin-induced hyperalgesia and the late phase of nociceptive response to formalin (Rupniak et al., 1997). In neuropathic pain models, B₁ peptide antagonists were also shown to inhibit hyperalgesia induced by peripheral nerve injury (Levy et al., 2000) and diabetes (Gabra and Sirois, 2002).

Bradykinin B₁ antagonists may have a therapeutic interest against chronic inflammatory diseases and inflammatory and neuropathic pain. Non-peptide antagonists with long-lasting efficacy and good bioavailability would be important tools to confirm the biological effects of B₁ inhibition, and possibly to develop new anti-inflammatory and analgesic drugs.

Here, we describe some general biochemical and pharmacological activities of a novel non-peptide B₁ antagonist, SSR240612 (2R)-2-[((3R)-3-(1,3-benzodioxol-5-yl)-3-[(6-methoxy-2-naphthyl)sulfonyl]amino)propanoyl]amino]-3-(4-([2R,6S)-2,6-dimethylpiperidinyl]methyl)phenyl)-N-isopropyl-N-methylpropanamide hydrochloride (Fig. 1).
Materials and methods

Binding assays

Assay of $[^3H]$Lys$^0$-desArg$^9$-BK binding to B₁ receptors

MRC5 human fibroblasts (American Type Culture Collection) and transfected HEK-293 cells expressing human B₁ receptors were routinely grown in Dulbecco’s modified medium with Glutamax-I (Life Technologies, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Life Technologies) and antibiotics. MRC5 were incubated for 4 h in DMEM containing 0.5 µg/ml interleukin-1β (Il-1β) to induce B₁ receptor synthesis. Cells were scraped and homogenized for 1 min using a Polytron (setting 8) in 25 mM TES-HCl containing 1 mM 1-10 phenantroin. Homogenates were centrifuged at 40000 g for 15 min at 4 °C, and pellets were resuspended in the same buffer, using Polytron (setting 8) for 1 min. Membranes were pelleted at 40000 g for 10 min at 4 °C, resuspended in the same buffer and conserved at -80°C.

$[^3H]$Lys$^0$-desArg$^9$-BK binding to cell membranes was performed in binding buffer of the following composition: 137 mM NaCl, 5.4 mM KCl, 1.05 mM MgCl₂, 1.8 mM CaCl₂, 1.2 mM NaH₂PO₄, 15.5 mM NaHCO₃, 10mM HEPES, 1 g/l bovine serum albumin (BSA), 140 mg/l bacitracin, 1 µM captopril, pH 7.4. Membranes were incubated for 30 min at 25 °C in 500 µl of binding buffer containing 1 nM $[^3H]$Lys$^0$-desArg$^9$-BK for competition curves or 0.1 to 10 nM for saturation isotherms. The reaction was terminated by filtration using a Brandel Harvester onto GF/B Whatman filters previously soaked for 2 h in 0.1 % polyethyleneimine. Filters were washed 3 times with 5 ml of binding buffer, and radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined by the addition of 1 µM of unlabeled Lys$^0$-desArg$^9$BK.
Assay of \[^{3}H\]BK binding to guinea-pig ileum homogenates (B\(_{2}\) Receptors)

The binding assay was performed according the method described by Manning et al (1986). Male Hartley guinea-pigs (Charles River) were killed by exsanguination under anesthesia. A 3 cm section of ileum was removed and homogenized in ice-cold buffer (25 mM TES, 1 mM 1-10 phenanthroline, pH 6.8) for 30 s using a Polytron (setting 6). Homogenates were filtered through three layers of gauze, and centrifuged at 40000 g for 10 min. Pellets were resuspended in the same buffer (Polytron 30 s setting 6) and the membranes were pelleted at 40000 g for 10 min and stored at –80 °C. \[^{3}H\]BK binding to ileum membranes was performed at 25 °C for 90 min in 2 ml of binding buffer (25 mM TES, 1 mM 1-10 phenanthroline, 1 g/l BSA, 140 mg/l bacitracin, 1 µM captopril, pH 6.8). Radioligand (0.1 nM) was incubated with membranes and varying concentrations of SSR240612 or BK. The incubation was terminated by filtration through GF/C Whatman filters previously soaked for 4 h in 0.1% polyethyleneimine. Filters were washed 3 times with 5 ml of binding buffer and the radioactivity was measured by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 1 µM unlabeled BK.

Assay of \[^{3}H\]BK binding to human recombinant B\(_{2}\) receptor membrane preparations.

Membrane preparations from a stable CHO-K1 cell line expressing the human recombinant B\(_{2}\) receptor were purchased from Euroscreen (Brussels, Belgium). Binding assays were performed at 4 °C for 45 min in 250 µl of 25 mM potassium phosphate buffer pH 6.6, 0.2% BSA containing radioligand (0.46 nM), membranes (2 µg protein) and varying concentrations of SSR240612. The incubation was terminated by filtration through GF/C Whatman filters previously soaked for 4 h in 0.1% polyethyleneimine. Filters were washed 3 times with 5 ml of binding buffer and radioactivity was measured by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 1 µM unlabeled BK.
Calculation and statistical analysis

Equilibrium constant (Kd) and concentration of receptor sites (Bmax) values were determined from Scatchard plots. Data from equilibrium binding (Kd, Bmax) and competition experiments (IC50) were analyzed using a non-linear least squares method (RS1). Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. Inhibition constant (Ki) values were calculated from the IC50 values using the Cheng and Prusoff equation (Cheng and Prusoff 1973).

Specificity studies

In addition, the affinity of SSR240612 for various receptors and channels and its effects on various enzyme activities were tested by CEREP (Celles l’Evescault, France).

In vitro functional assays

Measurement of inositol phosphate accumulation in MRC5 fibroblasts

[3H]inositol phosphate1 accumulation was measured in MRC5 fibroblasts labeled with [3H]myoinositol according to the method described by Oury-Donat et al.(1993) Cells cultured in six-well plates were labeled for 48 h with 5 µCi/ml [3H]myoinositol added to the culture medium (Dulbecco’s modified medium DMEM). Cells were then incubated for 4 h in DMEM containing 0.5 µg/ml II-1β to induce B1 receptor synthesis. Agonist stimulation of inositol phosphate 1 accumulation was performed in DMEM containing 50 mM LiCl, and test compounds. Antagonists were added 15 min before 10 nM Lys0-desArg10BK. After 30 min of incubation at 37 °C, the medium was discarded, and the reaction was stopped by rapid addition of 1 ml of cold methanol/HCl 1 N (vol/vol). The cells were scraped and the suspension was transferred to a glass tube with 1 ml chloroform and 20 µl HCl 12 N. The
aqueous phase was neutralized by 350 µl of 1 M NaHCO$_3$ and applied to 1 ml Dowex AG1X8 columns. $[^{3}H]$inositol phosphate 1 was eluted with 0.2 M ammonium formate 0.1M formic acid. Radioactivity was measured by liquid scintillation spectrometry.

**Isolated organ experiments**

$B_1$ antagonistic properties of SSR240612 were tested on desArg$^9$-BK induced contractions of rabbit aorta and myenteric plexus of rat ileum.

All experiments were performed in isolated organ baths containing an oxygenated (95% O$_2$-5% CO$_2$) and thermostated (37 °C) Krebs-modified physiological salt solution (KMPSS), pH 7.4.

Strips of rabbit aorta and myenteric plexus of rat ileum were prepared for tension recording. Tissues were rapidly excised from male albino rabbits (3-3.5 kg) and male Sprague-Dawley rats (100-150 g). The rat ileum was threaded into glass rods and the mesenteric attachment was stroked tangentially with cotton to separate the longitudinal muscle and adherent myenteric plexus from the circular muscle.

Tissues were allowed to equilibrate under a resting tension (2g for spirally cut strips from rabbit thoracic aorta, 0.5g for rat myenteric plexus) and and preincubated 20 to 22 H (rabbit) or 4 h (rat ileum) in KMPSS. Tension equivalent to the maximum response was obtained with KCl 100 mM for rabbit aorta and 40 mM for rat ileum.

Changes in tension were recorded isotonically with a length-displacement transducer (Ugo Basile, Italy). Cumulative concentration-response curves were obtained in the absence and then, after repeated washings, in the presence of a fixed concentration of SSR240612 added 45 min (rabbit aorta) or 1 h (rat ileum) before the agonist. Each preparation was exposed to one concentration of antagonist.
The agonist concentration producing 50% of the maximal effect (EC_{50}) was calculated using a four-parameter logistic model according to Ratkovsky and Reedy (1986), with adjustment by non-linear regression using the Levenberg-Marquardt algorithm in RS/1 software. The pA_{2} for antagonists, as defined by Arunkashana and Schild (1959), was obtained from linear regression of mean values of the log (dr-1) versus the negative log of the antagonist concentration. Computer analysis was done as described by Tallarida and Murray (1987).

**In vivo assays**

All procedures have been approved by the Animal Care and Use Committee of Sanofi-Synthelabo Recherche and were carried out in accordance with French legislation, the European Community Guidelines (86/609/EEC) and the declaration of Helsinki.

*Des Arg^{9}-BK induced mice paw edema*

Groups of 8 male albino mice under isoflurane anesthesia received a 20 µl intraplantar injection into the right hind paw of 5 µg of Il-1β in phosphate buffered saline (PBS)/bovin serum albumin (BSA) 0.1 %. Forty min later (T=0), mice received under anesthesia a 20µl intraplantar injection in the same paw of desArg^{9}- BK (10 µg/paw) in water.

SSR240612 or vehicle (5% v/v ethanol, 5% v/v tween 80 in water) was administered by oral route at the doses of 1, 3, and 10 mg/kg, 1 h before desArg^{9}- BK injection, and by intraperitoneal route at the doses of 0.1, 0.3, and 1 mg/kg 40 min before desArg^{9}- BK injection. Paw volume was measured with a plethysmometer at T = -2 h (initial measurement) and at several times after edema induction (T= 20, 40, 60 and 120 min). Paw edema volume was expressed in ml as the difference between the paw volume at each time
after edema induction and the initial paw volume. Results for each group are expressed as mean ± S.E.M. of individual paw edema volumes. Statistical analysis was performed after verification of normality and homogeneity of variances using ANOVAREP then Duncan test; treated groups versus desArg⁹-BK control group.

Capsaicin ear edema
Groups of 8 male albino mice, 28-33 g (Iffa Credo, France) were anesthetized with isoflurane. Capsaicin-ear edema was induced by applying a 25 mg/ml capsaicin solution in absolute ethanol using a micropipette to the right ear (10 µl to both the dorsal and ventral surfaces) of each animal. Thirty minutes later, animals were again anesthetized using isoflurane and killed by cervical dislocation. Right (treated) and left (untreated) ears of each animal were removed by cutting horizontally across the indentation at the base of the ear. Ear weights were determined to the nearest tenth of mg and the difference between the weights of the right and left ear (ear edema) were calculated for each animal.

SSR240612 at 0.3, 3 or 30 mg/kg or its vehicle (5% (v/v) Tween 80 and 5% (v/v) ethanol in water) were administered by the oral route under a 20ml/kg volume 1 h before capsaicin edema induction. Dexamethasone-21-acetate, used as the reference compound, was administered at 1 mg/kg (as the salified compound) using the same route and volume of administration. Results for each group are expressed as means ± S.E.M. of individual ear edema. Statistical analysis was performed after verification of normality and homogeneity of variances using ANOVA then Duncan test, treated group versus capsaicin control group.

Splanchnic artery occlusion/reperfusion
Male Sprague-Dawley rats (160-180 g) were fasted overnight before surgery. Animals were anesthetized with ketamine (0.3 ml i.p. of twice diluted SBH-Ketamin inj. 100 mg/ml) –
xylazine (0.3 ml i.m. of twice diluted Rompun 2%). After midline laparatomy, the superior mesenteric artery was isolated near its aortic origin. The artery was ligated with surgical thread for 30 min. During this period the intestinal tract was maintained at 37 °C by placing it between gauze pads soaked with warmed saline solution. After 30 min the ligature was removed and a 45 min reperfusion period was applied. Sham operated animals were subjected to the same surgical procedure except that the artery was not occluded. SSR240612 (1% v/v tween 80 in water) was injected in a volume of 1 ml/kg into the jugular vein 5 min before removal of the ligature. Sham and control animals received the vehicle.

After the 45 min reperfusion period, the animals were killed by an overdose of urethane. The small bowel was isolated between pylorus and sacculus rotundus. The degree of the tissue damage was estimated by the reduction of the weight of intestine. The intestine was gently cleaned from all adherent soft and adipose tissues. The bowel was cut up longitudinally along the axis and all components were removed from it by rinsing in saline. Wet weight of the tissue was measured using analytical scales.

Neutrophil accumulation in intestinal tissue was evaluated by measuring tissular activity of myeloperoxidase (MPO). Tissues were frozen in liquid nitrogen immediately after dissection and stored at –20 °C before activity determination. Tissue samples were thawed and homogenized for 1 min in 15 ml of 50 mM potassium-phosphate buffer (pH 6.0) containing 0.5 % (w/v) hexa-decyl-trimethyl-ammonium-bromid (HETAB, Sigma). The samples were frozen and thawed, then sonicated for 1 min. This was repeated once more. After the second sonication, samples were centrifuged for 30 min at 20000 g at 4 °C. An aliquot (0.01 ml of four times diluted sample) of the supernatant was then allowed to react with the solution containing 0.44 mM tetramethylbenzidine (TMB dihydrochloride, Fluka) and 0.0033% H₂O₂. Absorbance change was measured at 650 nm by spectrophotometry.
 Values are given as mean ± S.E.M. The significance of differences compared to the control group was obtained after performing the normality test and investigating homogeneity of variances (Bartlett test) using Dunnett’s test in the case of intestinal weights and Kruskal-Wallis test (followed by Mann-Whitney test) for MPO determinations. P<0.05 was considered as significant. All statistical analyses were performed using RS/1 software.

Ultraviolet irradiation-induced hyperalgesia in rat paw

The protocol used is according to Perkins and Kelly (1993).

Briefly, under light anesthesia (12 mg chloral hydrate + 1.9 mg pentobarbitone / ml), groups of 9 to 20 male OFA rats (100-150 g, Iffa Credo, France) were exposed to ultraviolet light (maximal intensity = 6210mJ/cm2) on the plantar surface of the left hind paw (UV exposed paw) for 20 min (the right hind paw being protected = non-exposed paw). Forty-eight hours later, the time taken to withdraw (withdrawal latency) each hind paw was measured, following a heat stimulus (a focused radiant heat beam, Ugo Basile unit) applied to the underside of the paw. The hind paws were exposed to the heat stimulus in a random manner so that there was no consistent order of testing between exposed and non-exposed paws and no rats had both paws exposed to heat stimulus immediately after each other. A cutoff time at 22 s was used to prevent blistering.

SSR240612 (suspended with 0.1% Tween 80 in saline) was administered by the oral route in a volume of 20 ml/kg 2 h before the thermal hyperalgesia measurement. In the time-course study, the compound was administered at 3 mg/kg p.o. 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h before measuring the withdrawal latencies in rats.

Results are expressed in seconds as mean withdrawal latencies (s) ± S.E.M. and statistical analyses were performed using a 2 way-ANOVA followed by Dunnett’s test.
Nociceptive behavior elicited by intraplantar injection of formalin in mice.

The protocol used was identical to the one described by Rupniak et al. (1997).

Groups of 10-12 male OF1 mice (30-35 g, Iffa Credo, France) received an intraplantar injection of formalin (20µl of 2.5% saline solution) into one hind paw. Then, mice were placed in a cylindrical glass (15 cm height x 10 cm diameter), and the duration of licking directed at the injected paw was recorded during the late phase (30-40 minutes) period after injection of formalin.

SSR240612 was suspended with 0.1% Tween 80 in saline and was administered by oral route in a volume of 5 ml/kg, 2 h before formalin administration. Results are expressed in seconds as mean licking time (s) ± S.E.M. and statistical analyses were performed using ANOVA followed by Dunnett’s test.

Neurogenic pain induced by sciatic nerve constriction in the rat

Male Sprague-Dawley rats (Iffa Credo, France) weighing 175-200 g on arrival were used. They were housed five to a cage, had free access to standard laboratory food and tap water and were kept on a 12-h light/dark cycle with light onset at 6 a.m. All animals were allowed to acclimatize to the housing facilities for at least 1 week before experiments.

The unilateral mononeuropathy was produced on the right hind paw according to the method of Bennett and Xie (1998) and Attal et al. (1990). Briefly, the animals were anesthetized with isoflurane (3%) followed by sodium pentobarbitone (50 mg/kg, i.p.). The common sciatic nerve was exposed by blunt dissection at the level of the mid-thigh and four ligatures (5-0 chromic catgut about 1 mm spacing) were placed around the sciatic nerve. After the surgery, the rats were housed in large cages with sawdust bedding to minimize possible painful mechanical stimulation. Non-operated rats were used as control.
Rats were used two weeks after surgery, a time period required to obtain a stable abnormal pain behavior (Bennett and Xie, 1998; Attal et al., 1990). Thermal nociception was tested by measuring the struggle latency elicited by immersion of the hind paw into a 46 °C water bath as extensively described (Attal et al., 1990; Lee et al., 1994; Idanpaan-Heikkila et al., 1997). For each rat, a control latency (mean of three consecutive trials, 45 min apart) was determined before administering the drug. The struggle latency was measured before (time 0) and 60, 120 and 180 min following either vehicle or SSR240612 oral administration at the dose of 10, 20 or 30 mg/kg. Data are expressed as mean ± S.E.M. (n = 9-21 rats per group) of struggle latencies (s). Control latency data from nerve injured and uninjured rats were analyzed by one-way ANOVA. Data from drug-treated animals were submitted to a 2-way ANOVA.

**Chemicals**

SSR240612 (Fig. 1) was synthesized at SANOFI-SYNTHELABO Recherche and was used as its hydrochloride salt. Radioactive ligands were purchased from Perkin-Elmer life Science (Paris, France). All peptides were obtained from Bachem (Budendorf, Switzerland).
Results

Receptor binding

As shown in Table 1, SSR240612 displaced the binding of $[^3]$H Lys$^0$-desArg$^9$-BK to membrane preparations of MRC5 human lung fibroblasts, and of HEK cells expressing human B$_1$ receptors with inhibition constants (Ki) of 0.48 nM and 0.73 nM, respectively. The affinity of SSR240612 for B$_2$ receptors labeled with $[^3]$H BK, was much lower with a Ki of 481 nM for guinea-pig ileum membranes and a Ki of 358 nM for membrane preparations of CHO cells expressing human B$_2$ receptors. The affinity of SSR240612 for human fibroblast MRC5 B1 receptors was higher than the affinity of agonist and antagonist peptides, Lys$^\cdot$desArg$^9$-BK (1.63nM), Lys-desArg$^9$,Leu$^8$-BK (2.44nM), desArg$^9$-BK (542nM) and desArg$^9$,Leu$^8$-BK (152.5nM) (Table 2). Saturation binding experiments were performed for the binding of $[^3]$H Lys$^0$-desArg$^9$-BK to MRC5 membranes in the absence and in the presence of SSR240612 (0.3, 1nM). Scatchard analysis of the data showed that increasing concentrations of SSR240612 produced an increase in dissociation constant (Kd) without significant modification in receptor density (Bmax) (Fig. 2), which is consistent with competitive antagonism.

In vitro functional studies

Antagonistic activity of SSR240612 at human B$_1$ receptors was studied by measuring inhibition of inositol phosphate-1 formation induced by Lys$^0$-desArg$^9$-BK (10 nM) activation of the B$_1$ receptor in MRC5 human fibroblast cells. Inositol phosphate 1 formation was concentration dependently inhibited by SSR240612 with an IC$_{50}$ of 1.9 nM. The drug itself did not modify the level of inositol phosphate 1, showing a total absence of agonistic effect. In contrast, SSR240612 was unable to antagonize inositol phosphate-1 formation induced by BK (3nM) activation of B$_2$ receptor in human fibroblast MRC5 (IC$_{50}$>1µM).
In rat ileum and rabbit aorta, SSR240612 caused a concentration-dependent rightward shift of the concentration-response curves for desArg\textsuperscript{9} - BK (Fig. 3 and 4). Schild plot analysis produced a pA\textsubscript{2} of 9.4 ± 0.1 and a slope of 1.5 ± 0.15 in the rat ileum, and a pA\textsubscript{2} of 8.9 ± 0.1 and a slope of 1.06 ± 0.11 in the rabbit aorta.

**In vivo studies**

The in vivo antagonistic effects of SSR240612 were first investigated on B\textsubscript{1} agonist-induced paw edema in mice after induction of B\textsubscript{1} receptors by intraplantar injection of IL-1\textbeta. SSR240612 significantly inhibited the desArg\textsuperscript{9} - BK induced paw edema in the mice, at the doses of 3 and 10 mg/kg p.o. and 0.3 and 1 mg/kg i.p. (Fig. 5).

The effects of SSR240612 were then studied on capsaicin-induced ear inflammation in mice. The drug administered per os 1 h before capsaicin significantly reduced the ear edema, but in a non concentration-dependent manner, as the three doses tested, 0.3, 3 and 30 mg/kg produced an inhibition of 43 %, 44% and 28% of the increase of ear weight (Fig. 6).

With regard to the post-ischemic intestinal reperfusion, the B\textsubscript{1} receptors, upregulated by ischemia, are supposed to contribute to systemic vasodilatation and intestinal injury consequent to neurogenic reflex induced by reperfusion (Madeddu et al. 2001). SSR240612 administered intravenously at 0.03 and 0.3 mg/kg dose-dependently inhibited the tissue destruction and neutrophil accumulation in the rat intestine, after splanchnic artery occlusion/reperfusion. While the lower dose (0.03 mg/kg) was inactive, the 0.3 mg/kg dose (i.v.) caused marked and significant reduction of mucosal damage (57% inhibition of tissue weight decrease, P<0.01) and neutrophil accumulation determined as MPO tissue activity (79% inhibition, P<0.01) (Table 3).
In the thermal hyperalgesia induced by UV irradiation in rats, SSR240612 dose-dependently and significantly increased the withdrawal latencies by 66 and 85 % at 1 and 3 mg/kg p.o., respectively with F(3,70)=6.82, p<0.001, the comparison between UV exposed and non exposed hind paw showing a highly significant effect: F (1,70)=10.15, p<0.002 (Fig.7).

The time-course study of SSR240612 at 3 mg/kg p.o. upon the ultraviolet exposed hind paw showed a maximal and significant effect at 1 h post-injection witch was long lasting (>6 H) (Fig. 8).

In the formalin model of inflammation in mice, the duration of the late phase of paw licking (30-40 min post-formalin injection) was dose dependently attenuated by SSR240612 at 10 and 30 mg/kg (27% and 69%, respectively) the effect being statistically significant at 30 mg/kg with F(1,38)=11.37, p<0.001 and a ID$_{50}$ of 18.7 mg/kg (16.2-22.2, 95% confidence limits). (Fig. 9)

As previously reported (Lee et al., 1994; Idanpaan-Heikkila et al., 1997), sciatic nerve injury produced a significant decrease (-45 %, F$_{1,96}$ = 138.33, p < 0.01) in the struggle latency relative to control non-operated rats (Fig. 10). In nerve-injured rats, oral SSR240612 administration significantly increased struggle latencies as compared to values before drug treatment. Two-way ANOVA (treatment x time) revealed a significant effect of drug treatment (F$_{3,211}$ = 5.21, p< 0.01) and of time period (F$_{3,211}$ = 3.38, p<0.05). The antinociceptive effect of SSR240612 was observed at 120 and 180 min after drug administration at the dose of 20 (+44%, p<0.01, +38%, p<0.05, at 120 and 180 min, respectively) and 30 mg/kg p.o. (+ 74%, p<0.01 and + 57 %, p<0.05, at 120 and 180 min, respectively). In contrast, SSR240612 had no significant effect on struggle latencies in uninjured rats (treatment: F$_{2,171}$ = 1.87, p > 0.05; time: F$_{3,171}$ = 1.77, p>0.05).
Discussion

The present study demonstrates that SSR240612 is a high-affinity, subtype-selective bradykinin B₁ antagonist.

In binding experiments, SSR240612 inhibited binding of [³H] Lys⁰-desArg⁹BK to human B₁ receptors with an inhibition constant (Ki) in the nanomolar range showing an affinity of the same order as human receptor specific peptides (Lys-desArg⁹-BK and Lys-desArg⁹,Leu⁸-BK) and much higher than rat receptor specific peptides (desArg⁹-BK and desArg⁹,Leu⁸-BK). The selectivity for B₁ versus B₂ receptors was in the range of 500 to 1000 fold. Finally, SSR240612 was tested over 100 (mainly human) receptor-binding, ion channel-binding and enzyme assays including the following: receptors to adenosine (A₁, A₂A, A₃), adrenergic (α₁, α₂, β₁, β₂) dopamine (D₁, D₂), nicotinic, muscarinic (M₁, M₂, M₃, M₄, M₅), opiate (μ, κ, δ, ORL₁), serotonin (5-HT₁A, 5-HT₂A, 5-HT₂C, 5-HT₃, 5-HT₅A, 5-HT₆, 5-HT₇), angiotensin (AT₁, AT₂), neurokinin (NK₁, NK₂, NK₃), calcitonin gene-related peptide (CGRP), cholecystokinin (CCK₁, CCK₂), corticotropin releasing factor (CRF₁, CRF₂), galanin (GAL₁, GAL₂), neurotensin (NT₁), vasopressin (V₁A); hormones (glucocorticoid, estrogen, progesterone, testosterone); ion channels (sodium, calcium, potassium and chloride); cyclooxygenases (COX₁, COX₂); phosphodiesterases (III and IV); acetylcholinesterase. SSR240612, at concentrations up to 1 μM, was inactive (inhibition less than 50%), except in muscarinic M₂ receptor assay (IC₅₀ = 0.48 μM) (data not shown).

The potent antagonism by SSR240612 of B₁ receptors has been demonstrated in-vitro for several species in different functional assays. On the human receptors, SSR240612 potently inhibited B₁ receptor-mediated inositolphosphate-1 formation in human fibroblasts MRC-5. On animal receptors, SSR240612 inhibited desArg⁹-BK induced contractions of rabbit aorta and rat ileum with similar potencies. These results show that SSR240612 is a potent antagonist of human, rabbit and rat B₁ receptors.
In-vivo, SSR240612 was shown to exert a potent antagonism of edema induced by desArg⁹-BK in the mouse hind paw after induction of B₁ receptor by intraplantar injection of IL-1β. SSR240612 was active by the oral route at the doses of 3 and 10 mg/kg.

SSR240612 was then studied in animal models where an enhanced endogenous B₁ specific tone is suspected to play a major role. B₁ receptor activation is known to induce pro-inflammatory effects including leucocyte accumulation, edema and pain. In addition, B₁ receptors are mainly implicated in neurogenic inflammation processes. The B₁ antagonist desArg⁹,leu⁸-BK has been reported to inhibit the capsaicin-induced mouse ear edema by modulation of neurogenic inflammation (Mantione and Rodriguez., 1990). B₁ antagonists also block inflammation-like hypotension and plasma protein infiltration of the intestinal wall, observed during mesenteric post-ischemic reperfusion in the anaesthetized rat, presumably regulated by a neurogenic reflex response (Madeddu et al., 2001). Likewise SSR240612 caused a significant though not dose dependent reduction of capsaicin-induced mice ear edema at the oral doses of 0.3 to 30 mg/kg and of mucosal damages and neutrophil accumulation produced by post-ischemic reperfusion of mesenteric artery in the rat at the dose of 0.3mg/kg (intravenous route).

As B₁ receptors are known to be important mediators of inflammatory pain, SSR240612 was tested on two pharmacological models, UV-induced hyperalgesia in rat paw, and the late phase of formalin test in mice, where pain or hyperalgesia were induced by local tissue injury. SSR240612, given orally, inhibited UV-induced hyperalgesia at the dose of 1 to 3 mg/kg,. It significantly antagonized the second phase of formalin-induced pain, at the doses of 30 mg/kg (oral administration)

B₁ receptors have also been suspected to modulate hyperalgesia induced by peripheral nerve injury. At 14 days after chronic constriction injury of the rat sciatic nerve, an increased expression of B₁ receptor mRNA has been reported in the lumbar dorsal root ganglia.
ipsilateral to the injured nerve site, coinciding with an analgesic effect of B₁ antagonist (Levy et al., 2001). In the same model of neuropathic pain, after 10 days of sciatic nerve constriction in the rat, SSR240612 antagonized hyperalgesia in the ipsilateral paw, at the doses of 20 to 30 mg/kg.

In conclusion, SSR240612 is a highly potent, selective non-peptide antagonist of the bradykinin B₁ receptor. SSR240612 displayed high potency for the B₁ receptor in all species tested (rat, mouse, rabbit and human). SSR240612 was found orally active in several models of neurogenic inflammation and inflammatory pain. These results suggest that SSR240612 may have potential therapeutic interest for the treatment of chronic inflammation and pain and is a useful tool for further exploring the pathophysiological role of B₁ receptors.
Acknowledgments

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References


Cheng Y and Prusoff (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which cause 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.


responses in kinin B1 and B2 receptor knockout mice. *Neuropharmacol* *41*:1006-1012.


Rawlingson A, Gerard NP and Brain SD (2001) Interactive contribution of NK(1) and kinin receptors to the acute inflammatory oedema observed in response to noxious heat stimulation: studies in NK(1) receptor knockout mice. Br j pharmacol 134:1805-1813.


Legends for figures

Fig. 1. Chemical structure of SR240612 (2R)-2-[((3R)-3-(1,3-benzodioxol-5-yl)-3-[[6-methoxy-2-naphtyl)sulfonyl]amino]propanoyl)amino]-3-(4-[[2R,6S)-2,6-dimethylpiperidinyl]methyl]phenyl)-N-isopropyl-N-methylpropanamide hydrochloride.

Fig. 2. Scatchard analysis of specific [³H]-Lys-[des-Arg⁹]-Bradykinin binding to B₁ receptors of human fibroblast MRC5 membranes in the absence (control) and in the presence of SSR240612. Values are means of triplicates. This is a typical experiment that was repeated 3 times with similar results. The Bmax was of 31 fmol / mg protein, and the Kd was 1.1 nM in the absence of SSR240612 and 2.05 nM, 4.0nM in the presence of SSR240612 at concentrations of 0.3 nM and 1 nM.

Fig. 3. Concentration-response curves for desArg⁹-BK induced contraction of rat ileum in the absence and in the presence of increasing concentrations of SSR240612 from 0.3 nM to 30 nM. Values are means ± S.E.M. (n = 4 to 8)

Fig. 4. Concentration-response curves for des-Arg⁹BK induced contraction of rabbit aorta in the absence and in the presence of increasing concentrations of SSR240612 from 3 to 30 nM. Values are means ± S.E.M. (n = 4 to 6)

Fig. 5. Effect of SSR240612 on time-dependent desArg⁹-BK induced paw edema in mice sensitized by IL-1 β. Animals received an intraplantar injection of IL-1β (5ng) in the right hind paw forty min before the intraplantar injection of 10 μg of desArg⁹-BK in the same paw (T=0). SSR240612 was administered by oral route 1 h before desArg⁹-BK injection (A) or intraperitoneal route 40 min before desArg⁹-BK injection (B). Paw edema volume (ml) was expressed by the difference between the paw volume for each time after the injection of desArg⁹-BK and the paw volume at time = -20 min.
Fig. 6. SSR240612 effects on capsaicin-induced ear inflammation in mice. 10 µl of a 25 mg/ml capsaicin solution were applied to the dorsal and ventral surface of the right ear. Ears weight were measured 30 min after capsaicin application. Data represent the difference between the weights of right and left ear. SSR240612 was administered by oral route 1 h before capsaicin. Values are mean ± S.E.M. (n = 8); **: p<0.01 versus capsaicin (ANOVA Duncan test); $$$: p<0.001 (Student t test).

Fig. 7. Withdrawal latencies (mean ± S.E.M.) for both ultraviolet exposed and not exposed hind paws of rats following oral administration of SSR240612.

**= p<0.01 vs vehicle treatment for u.v. exposed hind paw.

Fig. 8. Time-course of withdrawal latencies (mean ± S.E.M.) for both ultraviolet exposed and not exposed hind paws of rats following oral administration of 3 mg/kg of SSR240612.

**= p<0.01 vs vehicle treatment for u.v. exposed hind paw

Fig. 9. Effects of SSR240612 on the late phase paw licking response (30-40min) induced by intraplantar injection of a 2.5% solution of formalin in mice.

*=p<0.05 vs vehicle treatment.

Fig. 10. Antinociceptive effect of SSR240612 in a rat model of peripheral neuropathy. The struggle latency to immersion of the nerve-injured (black symbols) or uninjured (white symbols) hind paw of rats into a hot (46°C) water bath was measured before (time 0) and 60, 120 and 180 min after SSR240612 oral administration. Data are mean ± S.E.M. (n = 9-21 rats per group) of struggle latencies expressed in second. ++: p < 0.01 vs uninjured hind paw; * p < 0.05, ** p < 0.01 vs time 0.
TABLE 1  Binding affinity (Ki) of SSR240612 on B₂ kinins receptors of guinea pig ileum membranes and CHO cells expressing human B₂ receptor, using [³H]bradykinin as ligand and B₁ receptors of human fibroblasts MRC5 and HEK cells expressing human B₁ receptors, using [³H]Lys-desArg⁹-BK as ligand. Values are means ± S.E.M. of data obtained from at least three independent experiments performed in triplicates.

<table>
<thead>
<tr>
<th></th>
<th>B₁Human</th>
<th>B₁ Human</th>
<th>B₂ Guinea pig</th>
<th>B₂ Human</th>
</tr>
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<tbody>
<tr>
<td>MRC5</td>
<td>0.48 ± 0.04</td>
<td>0.73 ± 0.06</td>
<td>481 ± 107</td>
<td>358 ± 92</td>
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<td>HEK-B₁</td>
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TABLE 2 Binding affinity of SSR240612 and peptide bradykinin B1 receptor agonists and antagonists on B1 receptor of human MRC5 fibroblast, using [$^3$H]Lys-desArg9-BK as ligand. Values are means ± S.E.M. of data obtained from at least three independent experiments performed in triplicates.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_i$ (nM)</th>
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<tbody>
<tr>
<td>SSR240612</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>desArg9-BK</td>
<td>542 ± 49</td>
</tr>
<tr>
<td>desArg9,Leu8-BK</td>
<td>152.5 ± 18</td>
</tr>
<tr>
<td>Lys-desArg9-BK</td>
<td>1.63 ± 0.29</td>
</tr>
<tr>
<td>Lys-desArg9,Leu8-BK</td>
<td>2.44 ± 0.13</td>
</tr>
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TABLE 3  Effect of SSR240612 on splanchnic artery occlusion/reperfusion induced intestinal tissue injury and neutrophil accumulation in rats. The degree of tissue damage was estimated by the reduction of the intestine weight. Neutrophil accumulation in intestinal tissue was investigated through myeloperoxidase (MPO) activity. Values are means ± S.E.M. obtained from 12 to 16 independent experiments.  $$ p<0.01 versus sham.  
** p<0.01 versus control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight of intestine (g)</th>
<th>MPO (deltaAU/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± S.E.M.  (n)</td>
<td>Mean ± S.E.M.  (n)</td>
</tr>
<tr>
<td>Sham</td>
<td>7.450 ± 0.197 (16)</td>
<td>401 ± 34 (16)</td>
</tr>
<tr>
<td>Control</td>
<td>5.454 ± 0.210 (16) $$</td>
<td>1030 ± 144 (16) $$</td>
</tr>
<tr>
<td>SSR240612 0.03 mg/kg (i.v.)</td>
<td>5.630 ± 0.206 (12)</td>
<td>1147 ± 172 (12)</td>
</tr>
<tr>
<td></td>
<td>0.3 mg/kg (i.v.) **</td>
<td>530 ± 50 (16) **</td>
</tr>
</tbody>
</table>
Fig. 1.
Fig. 2.

Control

[SSR240612] = 0.3 nM

[SSR240612] = 1.0 nM
Fig. 3.

The graph shows the %max contraction of 4.10^-2M KCl against the log of desArg^9-BK (M) concentration. Different concentrations of SSR240612 are indicated with various symbols and line styles, each with a corresponding number of samples (n). The data points are accompanied by error bars indicating the standard error of the mean.
Fig. 4.
Fig. 5.

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JPET #59527
Fig. 6.
Fig. 7.

Withdrawal latency (s)

SSR240612 mg/kg po

0 1 3

Ultraviolet exposed hind paw No-exposed hind paw

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Fig. 8.
Fig. 9.
Fig. 10.