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Pharmacological characterization and radioligand binding properties of a high-affinity, nonpeptide, bradykinin B_1 receptor antagonist

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Abstract

Compound A (*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}-2-[(2*R*)-1-(2-napthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide) is a member of a new class of aryl sulfonamide dihydroquinoxalinone bradykinin B_1 receptor antagonists that should be useful pharmacological tools. Here we report on some of the pharmacological properties of compound A as well as the characterization of [^{35}S]compound A as the first nonpeptide bradykinin B_1 receptor radioligand. Compound A inhibited tritiated peptide ligand binding to the cloned human, rabbit, dog, and rat bradykinin B_1 receptors expressed in CHO cells with K_i values of 0.016, 0.050, 0.56, and 29 nM, respectively. It was inactive at 10 μM in binding assays with the cloned human bradykinin B_2 receptor. In functional antagonist assays with the cloned bradykinin B_1 receptors, compound A inhibited agonist-induced signaling with activities consistent with the competition binding results, but had no antagonist activity at the bradykinin B_2 receptor. Compound A was also found to be a potent antagonist in a rabbit aorta tissue bath preparation and to effectively block des-Arg^9 bradykinin depressor responses in lipopolysaccharide-treated rabbit following intravenous administration. The binding of [^{35}S]compound A was evaluated with the cloned bradykinin B_1 receptors. In assays with human, rabbit, and dog receptors, [^{35}S]compound A labeled a single site with K_d values of 0.012, 0.064, and 0.37 nM, respectively, and with binding site densities equivalent to those obtained using the conventional tritiated peptide ligands. Binding assays with the cloned rat bradykinin B_1 receptor were not successful, presumably due to the low affinity of the ligand for this species receptor. There was no specific binding of the ligand detected in CHO cells expressing the human bradykinin B_2 receptor. In assays with the cloned human bradykinin B_1 receptor, the pharmacologies of the binding of [^{35}S]compound A and [^{3}H][Leu^9]des-Arg^{10}-kallidin were the same. The high signal-to-noise ratio obtained with [^{35}S]compound A will allow this ligand to be a very useful tool for future investigations of the bradykinin B_1 receptor.

Keywords: Bradykinin B_1 receptor; Antagonist; Radioligand

1. Introduction

The kallikrein–kinin system has been implicated in a broad range of physiological and pathophysiological processes. Bradykinin and kallidin (Lys^0-bradykinin) are proteolytically cleaved from their precursor kininogens in response to various stimuli and preferentially activate the

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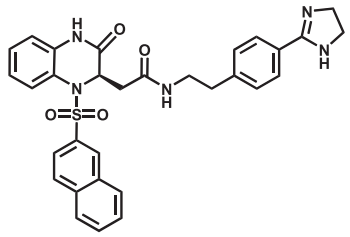
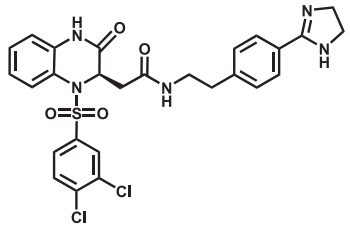
bradykinin B_2 receptor, although kallidin does have significant activity at the bradykinin B_1 receptor. These peptides can undergo further enzymatic processing to yield the corresponding carboxyl terminal des-arg kinins, des-Arg^9-bradykinin and des-Arg^{10}-kallidin, which act as selective agonists at the bradykinin B_1 receptor with very low affinity for bradykinin B_2 receptors (Regoli and Barabé, 1980; Regoli et al., 2001). The bradykinin B_1 and B_2 receptors are members of the seven transmembrane family of G-protein-coupled receptors and preferentially couple through the Gq pathway. These receptors were initially discriminated on the basis of pharmacological properties but have since been molecularly cloned from a number of species (Marceau et al., 1998). The bradykinin B_2 receptor is constitutively present in many tissues and subserves most of the acute cellular and tissue responses commonly associated with bradykinin. Responses to bradykinin B_1 receptor agonists are, for the most part, absent under normal circumstances but undergo induced expression following tissue trauma or exposure to inflammatory mediators such as interleukin-1ß or tumor necrosis factor-α. However, the hypotensive action of bradykinin B_1 receptor agonists in dog and their acute contractile actions in mouse fundus indicate that bradykinin B_1 receptor expression in all species may not be exclusively linked to inflammatory stimuli (Rhaleb et al., 1989; Nakhostine et al., 1993; Nsa Allogho et al., 1996).

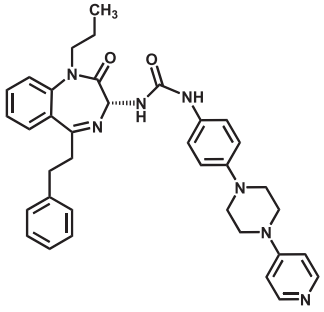
Bradykinin has long been recognized as a potent mediator of acute pain and inflammatory responses via activation of bradykinin B_2 receptors located on nociceptive neurons and in the vasculature. Over the last decade, evidence has also been obtained, indicating a role for the bradykinin B_1 receptor in inflammatory pain and, especially, in chronic inflammatory states (Perkins et al., 1993; Rupniak et al., 1997; Couture et al., 2001; Mason et al., 2002). This is consistent with the induced expression of the receptor produced by cytokines and tissue trauma. Peptide bradykinin B_1 receptor antagonists have been demonstrated to have antinociceptive activity in animal pain models, and studies with bradykinin B_1 receptor knockout mice have supported a role for the receptor in mediating pain responses (Pesquero et al., 2000; Ferreira et al., 2001; Ferreira et al., 2002). These findings have driven an interest in the development of bradykinin B_1 receptor antagonists as novel analgesic agents with the result that reports of nonpeptide antagonists have recently appeared (Horlick et al., 1999; Su et al., 2003; Wood et al., 2003; Gougat et al., 2004). Such agents should provide valuable pharmacological tools to further elucidate the physiological properties of the bradykinin B_1 receptor. Here, we describe some pharmacological properties of a highly potent, nonpeptide bradykinin B_1 receptor antagonist referred to herein as compound A (*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}-2-[(2*R*)-1-(2-napthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide), which is structurally based upon the dihydroquinoxalinone series of compounds reported by Su et al. (2003) (Fig. 1).



Compound A



Compound B



Compound C

Fig. 1. Structures of the nonpeptide bradykinin B_1 receptor antagonists used in the present studies.

2. Materials and methods

2.1. Chemicals

All peptides were obtained from Bachem Bioscience (King of Prussia, PA, USA) and prepared as 10-mM stock solutions in 0.1% acetic acid and stored at −20 °C. Compound A, compound B (2-{2*R*)-1-[(3,4-dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl}-*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl} acetamide), and compound C (*N*-[(3*R*)-2-oxo-5-(2-phenylethyl)-1-propyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]-*N*′ -[4-(4-pyridin-4-ylpiperazin-1-yl)phenyl]urea) were synthesized at Merck's Department of Medicinal Chemistry (West Point, PA, USA; Fig. 1). Compound B is compound 11 in the report of Su et al. (2003) and compound C is compound 13 in Wood et al. (2003). [^{35}S]compound A was prepared in Merck's Department of Drug Metabolism (Rahway, NJ, USA). The specific activity of different batches of the ligand varied from 1150 to 1250 Ci mmol^{-1}. [^{3}H][Leu^9]des-Arg^{10}-kallidin, [^{3}H]des-Arg^{10}-kallidin, and [^{3}H]bradykinin were from Perkin Elmer (Boston, MA, USA) and [^{3}H]myo-inositol was from Amersham Bioscien-

ces (Piscataway, NJ, USA). Probenecid, norepinephrine, and *Escherichia coli* lipopolysacharride (serotype 0111:B4) were obtained from Sigma-Aldrich (St. Louis, MO, USA), and fluo-3 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, USA). Cell culture media components were from GIBCO-BRL (Rockville, MD, USA).

2.2. Cell culture

The cloning of the human, rabbit, and dog bradykinin B_1 receptors in these laboratories has been described (Menke et al., 1994; MacNeil et al., 1995; Hess et al., 2001). The rat bradykinin B_1 receptor was similarly cloned and all receptors were stably expressed in CHO cells. The cloning of the human bradykinin B_2 receptor and expression in CHO cells have also been described previously (Hess et al., 1992). All lines were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in Iscove's modified Dulbecco's medium containing 10% heat-inactivated fetal bovine serum, antibiotics, and 1 mg ml^{-1} G418.

2.3. Animals

Male New Zealand White rabbits (2.3–2.6 kg) were obtained from Covance Research Products (Denver, PA, USA). All experimental procedures were approved by the Merck Institutional Animal Care and Use Committee and followed the guidelines of the National Institutes of Health (USA).

2.3.1. Radioligand binding assays

All binding assays were performed with receptors stably expressed in CHO cells. Membranes were used for assays with the human and rabbit bradykinin B_1 receptors and the human bradykinin B_2 receptor, while intact cells were used for the rat and dog bradykinin B_1 receptor assays. [^{3}H][Leu^9]des-Arg^{10}-kallidin was used for labeling the human and rabbit bradykinin B_1 receptors, [^{3}H]des-Arg^{10}-kallidin for the rat and dog bradykinin B_1 receptors, and [^{3}H]bradykinin for the human bradykinin B_2 receptor. Cells were detached from flasks with 1 mM EDTA in phosphate-buffered saline and pelleted at 1000×*g*. For the membrane binding assays, the cells were resuspended in 20 mM HEPES and 1 mM EDTA (pH 7.4; lysis buffer), homogenized with a Polytron, and centrifuged at 50,000×*g* for 20 min. The membrane pellets were rehomogenized in lysis buffer and centrifuged again at 50,000×*g*, and the final pellets were resuspended at 5 mg ml^{-1} membrane protein in assay buffer (120 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.4 at room temperature) with 1% bovine serum albumin and stored at −80 °C. On the day of assay, membranes were thawed, centrifuged at 14,000×*g* for 10 min, and then resuspended in assay buffer containing 100 μg ml^{-1} bacitracin and 0.1% bovine serum albumin. For all assays, competitors were diluted in dimethyl sulfoxide and 4 μl was added to assay tubes followed by the addition of 100 μl of radioligand and 100 μl of membrane suspension. Nonspecific binding for the bradykinin B_1 and B_2 receptor binding assays was determined using 1 μM des-Arg^{10}-kallidin and 1 μM bradykinin, respectively. Tubes were incubated at 22 °C for 90 min followed by filtration through polyethyleneimmine-treated glass fiber filters (0.3%, 3 h) using a Tomtec 96-well harvester. Assay buffer without bacitracin and bovine serum albumin was used for filter washing. Radioactivity retained by the filter was counted using a Wallac Beta-Plate scintillation counter. In the whole cell binding assays with the rat and dog bradykinin B_1 receptor expressing CHO cells, the cells were detached from plates, centrifuged at 1000×*g*, and washed one time by recentrifugation in assay buffer prior to assay. Otherwise, the binding procedure was identical to the membrane assay. Membrane and whole cell binding assays with [^{35}S]compound A were also performed in an identical fashion, except that incubations were carried out for 2 h. Competition data were analyzed using GraphPad Prism software. Inhibitor dissociation constants (K_i) were calculated from IC_{50} values using the relationship described by Cheng and Prusoff (1973). Analysis of saturation binding data was performed using the KELL program (Biosoft, Ferguson, MO, USA).

Association studies were performed at 20 pM [^{35}S]compound A at 22 °C. For dissociation experiments, [^{35}S]compound A was allowed to incubate with human bradykinin B_1 receptor CHO cell membranes for 2 h upon which 1 μM des-Arg^{10}-kallidin was added to prevent rebinding of the radioligand to the receptor. Aliquots from the incubation were filtered at time 0 and at selected time points over a 4-h period. A parallel set of incubations was performed in the presence of 1 μM des-Arg^{10}-kallidin to determine nonspecific binding at each time point.

2.3.2. Phosphoinositide hydrolysis

CHO cells grown in 175-mm^2 flasks were labeled overnight with 0.7 μCi ml^{-1} [^{3}H]inositol. After removing the medium, flasks were rinsed with phosphate-buffered saline and the cells detached with 1 mM EDTA in phosphate-buffered saline. Following centrifugation at 1000×*g*, cells were resuspended in a HEPES-buffered physiological solution (20 mM HEPES, 120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, pH 7.4) containing 10 mM LiCl, and 475-μl aliquots were preincubated for 15 min at 37 °C in the absence or presence of compound A. Twenty-five microliters of agonist was then added and reactions were terminated after 15 min by the addition of 0.4 ml of ice-cold 12% perchloric acid, 3 mM EDTA, and 1 mM diethylenetriamine pentaacetic acid. Following neutralization with a solution of 3 M KOH, 0.25 M 2-(*N*-morpholino) ethanesulfonic acid, and 0.25 M 3-(*N*-morpholino)propanesulfonic acid, [^{3}H]inositol monophosphate was recovered by ion exchange column chromatography with AG 1-8X resin

(Rio-Rad, Richmond, CA, USA) and measured by liquid scintillation counting (Schneck et al., 1994). Antagonist dissociation constants (K_B) were calculated using the dose ratio method: $R'/R=1+A/K_B$, where R and R' are the EC_{50} values for the agonist in the absence and presence of the antagonist, respectively, and A is the concentration of the antagonist.

2.3.3. Calcium mobilization

CHO cells expressing the bradykinin receptors were seeded at a density of 50,000 cells well^{-1} in a 96-well plate in 200 μl of Iscove's modified Dulbecco's medium containing 10% fetal bovine serum. After overnight incubation at 37 °C, the cell plates were washed twice with Hanks buffered salt solution and the cells were incubated for 60 min at 37 °C with Hanks solution containing 4 μM fluo-3 acetoxymethyl ester and 1 mM probenecid. The cells were then washed four times with dye-free salt solution containing probenecid, and 100 μl of salt solution with 1 mM probenecid was added to each well. Des-Arg^{10}-kallidin-induced elevation of cytosolic calcium was determined using a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA). All assays were conducted at 37 °C. An antagonist was added to the appropriate wells in a volume of 50 μl of Hanks solution 4 min prior to the addition of des-Arg^{10}-kallidin in a 50-μl volume. In each of the cell lines, a des-Arg^{10}-kallidin concentration producing 80% of the maximal obtainable response was used. Changes in cellular fluorescence due to increased cytosolic calcium ion concentrations in response to the agonist were determined using an excitation wavelength of 488 nm and a 510–570 nM bandwidth emission filter. Curve fitting and IC_{50} calculations were performed using GraphPad Prism software. At least eight concentrations of antagonist were used to generate each inhibition curve.

2.3.4. Rabbit aorta organ bath

In vitro contractile assays with rabbit thoracic aorta were modified from the methods of Regoli et al. (1978) and Bouthillier et al. (1987). Male New Zealand White rabbits were sacrificed with an overdose of pentobarbital administered intravenously via an ear vein following local anesthesia. The thoracic aorta was dissected, cleaned of extraneous adhering tissue, and spirally cut. The tissue was incubated at 37 °C overnight in 1 l of Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaCO_3$, and 5.5 mM D-glucose) oxygenated with 95% O_2–5% CO_2. Segments of tissue (1.5 cm) were mounted in 5-ml organ baths under $1\times g$ tension for isometric recording of contractions. Contractile responses to des-Arg^{10}-kallidin were recorded on a Gould 3800 strip chart recorder using Gould Statham force transducers. After 1 h of equilibration at 37 °C with buffer changes every 15 min, des-Arg^{10}-kallidin cumulative concentration–response curves were obtained for all tissues. The tissues were again washed for 1 h with buffer changes every 15 min before obtaining a second concentration–response curve to des-Arg^{10}-kallidin. When examined, compound A was present during the last 30 min of the second wash cycle. Data were analyzed using GraphPad Prism. For calculations, responses to des-Arg^{10}-kallidin in the second concentration–response curve were expressed as the percentage of the maximal response obtained in the previous concentration–response curve for each tissue. K_B values were derived as in Section 2.4.2.

2.3.5. Rabbit blood pressure

Des-Arg^9-bradykinin hypotensive responses in New Zealand White rabbits were studied following published methods (Regoli et al., 1981; Marceau et al., 1984). Bacterial lipopolysaccharide (10 μg kg^{-1}) prepared in saline was administered intravenously into an ear vein following topical anesthesia. After 3 h, the animals were anesthesized with pentobarbital (30 mg kg^{-1}, i.v.) via an ear vein using a 21-gauge butterfly infusion set connected to a three-way stopcock. Two syringes were attached for administering supplemental pentobarbital to maintain anesthesia and heparinized saline (100 U ml^{-1}) for flushing. A catheter was inserted into one femoral artery for recording of blood pressure using a disposable pressure transducer (Maxxim Medical, Athens, TX, USA) and a Modular Instruments (West Chester, PA, USA) data acquisition system. A femoral vein was cannulated for intravenous delivery of des-Arg^9-bradykinin and antagonists. Animals received nitro-L-arginine methyl ester (30 mg kg^{-1}, i.v.) to stabilize blood pressure over the course of the study as well as 1 mg kg^{-1} atropine. Two or three responses to 1 μg kg^{-1} des-Arg^9-bradykinin, a threshold maximally active dose, were obtained separated by 20 min to test the reproducibility of the peptide's depressor response. Subsequently, des-Arg^9-bradykinin responses were obtained at 30-min intervals with compound A administered 10 min before the agonist. Peptide antagonists were administered 2 min before des-Arg^9-bradykinin. The ID_{50} (antagonist dose producing 50% inhibition of the des-Arg^9-bradykinin response) values were generated from curve fitting of the data with GraphPad Prism.

3. Results

3.1. [^{3}H][Leu^9]des-Arg^{10}-kallidin and [^{3}H]des-Arg^{10}-kallidin competition binding assays

Compound A demonstrated a very high affinity for the cloned human receptor with a K_i of 16±5 pM (mean± S.E.M., n=3) in [^{3}H][Leu^9]des-Arg^{10}-kallidin competition binding studies. The K_i values obtained in binding assays with the cloned rabbit, dog, and rat bradykinin B_1 receptors were (nM; mean±S.E.M) 0.05±0.01, 0.56±0.09, and 29±10, respectively (Fig. 2). The K_i values for compound

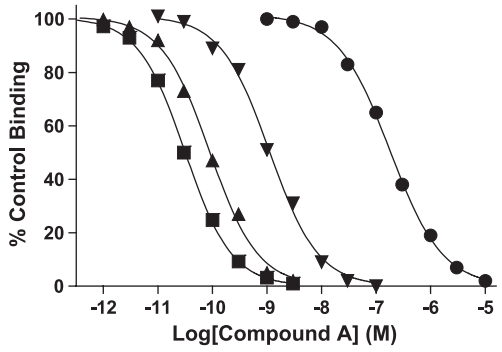


Fig. 2. Inhibition of [^{3}H][Leu^9]des-Arg^{10}-kallidin (human ■; rabbit ▲) or [^{3}H]des-Arg^{10}-kallidin (dog ▼; rat ●) binding to cloned bradykinin B_1 receptors. Assays were performed in triplicate at the indicated antagonist concentrations as described in Materials and Methods. Inhibitor dissociation values are given in the text. The results are from a single experiment that was repeated two additional times with similar results.

A were unchanged when human and rabbit bradykinin B_1 receptor binding assays were performed with the agonist radioligand [^{3}H]des-Arg^{10}-kallidin (data not shown). The slopes of the inhibition curves in all assays were near unity, consistent with a competitive mode of interaction between compound A and the kinin peptide radioligands. The antagonist ligand [^{3}H][Leu^9]des-Arg^{10}-kallidin performed poorly in binding assays with the rat and dog bradykinin B_1 receptor cells because of its relatively low affinity for these species receptors and the low receptor expression in these lines. The effect of 30 pM compound A was examined on [^{3}H][Leu^9]des-Arg^{10}-kallidin saturation binding at the human bradykinin B_1 receptor. There was no change in the density of sites labeled by [^{3}H][Leu^9]des-Arg^{10}-kallidin, but its apparent affinity was reduced by a factor of four, which is consistent with a competitive mode of interaction. Compound A had no effect on [^{3}H]bradykinin binding to the cloned human bradykinin B_2 receptor at concentrations up to 10 μM.

3.2. CHO cell antagonist assays

Des-Arg^{10}-kallidin stimulated phosphatidyl inositol hydrolysis in the human bradykinin B_1 receptor CHO cells with an EC_{50} of 0.3 nM and maximally elevated [^{3}H]inositol monophosphate levels about fourfold over basal (data not shown). At a concentration of 0.5 nM des-Arg^{10}-kallidin, the IC_{50} for compound A inhibition of the des-Arg^{10}-kallidin response was 0.032±0.011 nM (n=3). Compound A had no discernible agonist effect when tested at concentrations up to 1 μM, nor did the compound have any effect on basal production of [^{3}H]inositol monophosphate in the human bradykinin B_1 receptor CHO cells. However, we have no evidence for constitutive bradykinin B_1 receptor activity in these cells, and the lack of inhibition of basal signaling does not dismiss the possibility that this compound may have inverse agonist activity. The effect of compound A on des-Arg^{10}-kallidin dose–response curves was examined. Increasing concentrations of compound A produced a progressive shift to the right of the des-Arg^{10}-kallidin curve, but also depressed the maximum obtainable response to the agonist (Fig. 3). The antagonist dissociation constants calculated from the data using the dose ratio method were 21, 23, and 32 pM for the three concentrations of compound A examined. At 1 μM, compound A had no effect on bradykinin-induced phosphatidyl inositol hydrolysis in human bradykinin B_2 receptor CHO cells.

The antagonist activity of compound A was compared between species in calcium mobilization assays with the cloned bradykinin B_1 receptors. The IC_{50} values obtained for the human, rabbit, and dog bradykinin B_1 receptor-expressing cells were (nM; n=3) 0.04±0.012, 0.14±0.03, and 3.5±1.5, respectively. The response in the rat line was too poor to generate accurate data. There was no agonist activity of compound A detected in any of the cell lines.

3.3. Rabbit aorta tissue bath

Helical strips of rabbit aorta incubated overnight developed contractile responses to des-Arg^{10}-kallidin. The responses were pharmacologically confirmed to be mediated by the bradykinin B_1 receptor using several peptide bradykinin B_1 and B_2 receptor antagonists (data not shown). Compound A produced a dose-dependent shift of the des-Arg^{10}-kallidin concentration–response curve, although with a depression of the maximum response (Fig. 4). The K_B values obtained for compound A when tested at 0.3 and 1 nM concentrations were 78 and 105 pM, respectively. These values are consistent with the activity of compound A in the [^{3}H][Leu^9]des-Arg^{10}-kallidin competition binding studies with the cloned rabbit bradykinin B_1 receptor and the calcium mobilization assay results. Compound A at 1 μM

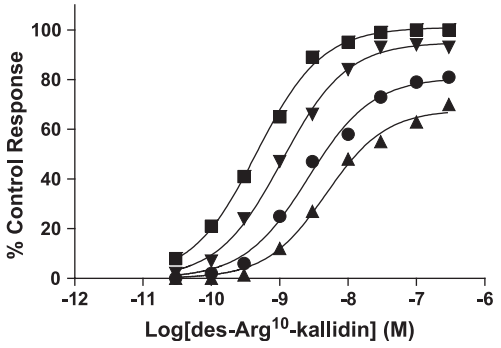


Fig. 3. The effect of compound A on des-Arg^{10}-kallidin-stimulated accumulation of [^{3}H]inositol monophosphate in CHO cells expressing the human bradykinin B_1 receptor. Des-Arg^{10}-kallidin concentration–response curves were obtained in the absence (■) of compound A and in the presence of 30 (▼), 100 (●), and 300 pM (▲) concentrations of the antagonist. The data are plotted as a percentage of the maximum response obtained to des-Arg^{10}-kallidin in the absence of compound A. The experiment was performed in triplicate as described in Materials and Methods and was repeated two additional times with similar results.

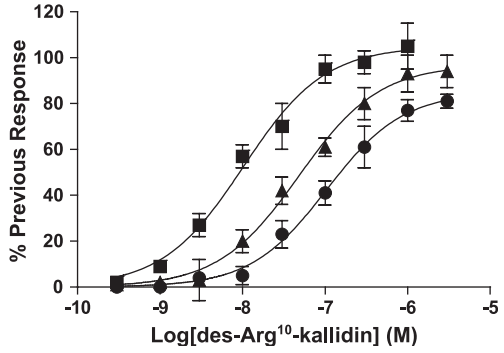


Fig. 4. The effect of compound A on des-Arg^{10}-kallidin-induced contraction of the isolated rabbit aorta. The results are from one experiment using three tissues for each condition. Des-Arg^{10}-kallidin dose–response curves were obtained in the absence (■) of compound A and in the presence of 300 (▲) and 1000 pM (●) concentrations of the antagonist. The data are plotted as a percentage of the maximum response to des-Arg^{10}-kallidin from the first dose–response curve for each tissue performed in the absence of compound A. Data points represent mean±S.E.M. The experiment was repeated with similar results.

had no effect on norepinephrine-induced contractions of rabbit aorta (data not shown).

3.4. In vivo antagonism in lipopolysaccharide rabbit

Lipopolysaccharide administration to rabbit has been shown to induce depressor responses to bradykinin B_1 receptor agonists. In the studies reported here, des-Arg^9-bradykinin (1 μg kg^{-1}, i.v.) produced relatively consistent decreases of 25–30 mm Hg in mean arterial pressure within 4 h after lipopolysaccharide administration. The nature of the responses was confirmed by the use of peptide bradykinin B_1 and B_2 receptor antagonists. The ID_{50} value for the bradykinin B_1 receptor antagonist [Leu^9]des-Arg^{10}-kallidin was 2.8 μg kg^{-1}, while the selective bradykinin B_2 receptor antagonist Hoe 140 was inactive at doses up to 30 μg kg^{-1} (Fig. 5). Compound A inhibited the des-Arg^9-bradykinin-induced decrease in blood pressure with an ID_{50} of 1.1 μg kg^{-1} (Fig. 5). It had no effect on baseline blood pressure at the highest dose tested.

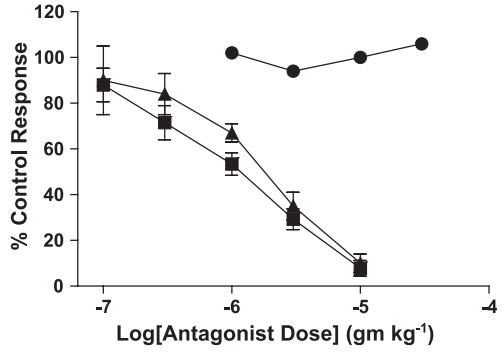


Fig. 5. Inhibition of des-Arg^9-bradykinin-induced depressor responses in lipopolysacharride-treated rabbit by compound A (■; n=4), [Leu^9]des-Arg^{10}-kallidin (▲; n=3), and Hoe 140 (●; n=2). The data points are mean±S.E.M.

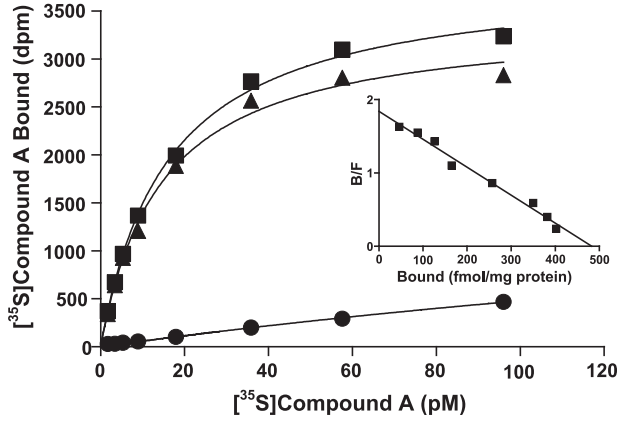


Fig. 6. Saturation binding of [^{35}S]compound A to human bradykinin B_1 receptor CHO cell membranes. Represented in the hyperbolic analysis are total (■), nonspecific (●), and specific (▲) binding of the radioligand. Scatchard transformation of the specific binding is shown in the inset. The assay was performed in triplicate at the indicated concentrations of [^{35}S]compound A and was repeated two additional times with similar results.

3.5. [^{35}S]Compound A binding to bradykinin B_1 receptors

At 22 °C in membranes prepared from the human bradykinin B_1 receptor CHO cells, [^{35}S]compound A binding monophasically reached equilibrium with a $t_{1/2}$ of 23±6 min and dissociated slowly from a single site with a $t_{1/2}$ of 182±12 min (n=3; data not shown). The association and dissociation rate constants were 0.87 min^{-1} nM^{-1} and 0.0038 min^{-1}, respectively, yielding a derived K_d of 4.4 pM. In saturation binding studies with the cloned human bradykinin B_1 receptor membranes, [^{35}S]compound A labeled a single set of sites with K_d=12±4 pM (n=3) and a binding density equivalent to that labeled by [^{3}H][Leu^9]des-Arg^{10}-kallidin (Fig. 6). There was no specific binding of [^{35}S]compound A to membranes in the human bradykinin B_2 receptor CHO cells. Competition binding studies with the cloned human bradykinin B_1 receptor indicated that the pharmacologies of [^{35}S]compound A and [^{3}H][Leu^9]des-Arg^{10}-kallidin were similar (Table 1), and the slopes of the curves for both peptide and nonpeptide competitors using either radioligand were near unity.

Table 1
Affinities of peptide and nonpeptide compounds for the cloned human bradykinin B_1 receptor determined using either [^{3}H][Leu^9]des-Arg^{10}-kallidin or [^{35}S]compound A as radioligand

	K_i (nM)	
	[^{3}H][Leu^9] des-Arg^{10}-kallidin	[^{35}S] compound A
[Leu^9]des-Arg^{10}-kallidin	0.17±0.037	0.24±0.049
Des-Arg^9-bradykinin	141±34	228±45
Bradykinin	>1000	>1000
Compound A	0.016±0.005	0.014±0.003
Compound B	0.034±0.005	0.025±0.005
Compound C	0.56±0.18	0.39±0.15

The results are mean±S.E.M. from at least three determinations for each inhibitor.

Compound B is the 3,4-dichlorophenyl analog of compound A reported by Su et al. (2003) (Fig. 1) and compound C is a benzodiazepine bradykinin B_1 receptor antagonist described by Wood et al. (2003) (Fig. 1). [^{35}S]compound A also bound to a single site in CHO cells expressing the rabbit and dog receptors with K_d values of (nM; n=3) 0.064±0.01 and 0.37±0.12, respectively. These affinities are consistent with the K_i values of compound A obtained in the tritiated peptide competition studies. Binding studies with the rat bradykinin B_1 receptor CHO cells did not yield interpretable data, presumably due to the low affinity of compound A for this receptor.

4. Discussion

The discovery of high-affinity and selective nonpeptide bradykinin B_1 receptor antagonists will advance the understanding of the physiological roles of the kinin system. In addition to being much needed pharmacological tools, these compounds can potentially allow the development of high specific activity radioligands for receptor studies. Following the development of the phenyl sulfonamide dihydroquinoxalinone bradykinin B_1 receptor antagonists reported by Su et al. (2003), other aryl substituents that would be more amenable to the introduction of sulfur-35 into the structure were considered. Consequently, the bradykinin B_1 receptor antagonist activity of the 2-napthyl sulfonamide (compound A) was investigated. As with the previously reported phenyl compounds, compound A exhibited the highest affinity for the human and rabbit bradykinin B_1 receptors in competition binding studies, the lowest affinity for the rat receptor, and an intermediate—although still significant—affinity for the dog receptor. Earlier studies on the activity of kinins at human, rabbit, and rodent bradykinin B_1 receptors had already suggested the potential for divergent pharmacologies at different species of bradykinin B_1 receptors Hess et al., 1996; MacNeil et al., 1997; Regoli et al., 1997).

In vitro functional assays demonstrated that compound A was a selective bradykinin B_1 receptor antagonist. In calcium mobilization assays with the cloned bradykinin B_1 receptors, compound A exhibited antagonist activities that reflected the binding assay data. It was also important to document the antagonist activity at a native receptor in tissue, and the isolated rabbit aorta has been a commonly used preparation for studies of bradykinin B_1 receptor pharmacology. Indeed, it was from studies with this tissue that the existence of a second type of bradykinin receptor that was preferentially activated by carboxyl terminal des-Arg kinins was originally proposed (Regoli et al., 1978). In contractile studies with aorta, compound A produced a concentration-dependent shift in the des-Arg^{10}-kallidin dose–response curve, although there was a reduction in the maximum agonist response. The reduced maximum response is likely due to slow dissociation of the antagonist from the receptor. A similar reduction in response was observed in the phosphatidyl inositol turnover studies with the cloned human receptor cell. Other compounds related to compound A, but with lower affinity for the rabbit receptor and presumably faster dissociation rates, have been examined in the isolated aorta preparation with no reduction in the maximum agonist response in the same types of studies. Also, compound A had no effect at 1 μM on the rabbit aorta response to norepinephrine, which contracts the tissue through the same signal transduction pathway used by the bradykinin B_1 receptor. The in vivo activity of compound A was tested in the anesthesized lipopolysacharride rabbit preparation and found to potently antagonize the depressor responses to des-Arg^9-bradykinin. The selectivity of the antagonism was not evaluated, but other structurally related compounds that are potent bradykinin B_1 receptor antagonists, including the 3,4-dichlorophenyl sulfonamide (compound B), have been tested against bradykinin-induced decreases in blood pressure and found to be inactive at doses that fully inhibit des-Arg^9-bradykinin responses. With respect to the breadth of selectivity of this class of structures, both compound A and compound B have been examined at a commercial screening facility (MDS Pharma Services, Bothell, WA, USA). Compound B had no significant activity (i.e., >1 μM) at any of over 170 other G-protein-coupled receptors, transporters, and enzymes, whereas compound A showed submicromolar affinity in radioligand binding assays at two muscarinic receptor subtypes with K_i values of about 0.5 μM and at a guinea pig serotonin receptor where it inhibited binding with a K_i of 55 nM. Functional agonism or antagonism at these receptors was not addressed.

Compound A was radiolabeled for evaluation of its potential use as a novel bradykinin B_1 receptor ligand. Saturation binding studies with the cloned human, rabbit, and dog receptors demonstrated that [^{35}S]compound A bound to a single class of sites with affinities predicted from the competition studies using the peptide radioligands. It can be seen in the data depicted in Fig. 6 that specific binding with the ligand is very high. Compound A has a log P value of 0.25, which probably contributes to the low nonspecific binding of the radioligand. In competition studies, kinin peptide, a structurally unrelated nonpeptide antagonist (compound C), as well as the dicholorophenyl analogue of compound A (compound B) appeared to behave competitively with [^{35}S]compound A as judged from the slope of the competition curves using the human bradykinin B_1 receptor membranes. Although not presented here, competition binding assays with the cloned rabbit and dog bradykinin B_1 receptors yielded similar results. It is unfortunate that this series of compounds has the lowest affinity for the rat bradykinin B_1 receptor of the species examined because of the widespread use of the rat for pharmacological and physiological investigations. Attempts to use [^{35}S]compound A for localization of native receptors in rabbit and nonhuman primate tissues are currently underway. [^{35}S]compound A has been successfully used to

label the human bradykinin B_1 receptor in in vitro studies in a transgenic rat line (Hess et al., 2004). Because of its high specific activity and favorable binding properties, the radioligand allowed the development of an ex vivo binding assay to assess central nervous system receptor occupancy of peripherally administered nonpeptide bradykinin B_1 receptor antagonists. [^{35}S]compound A, as well as the unlabeled compound, should be valuable tools in future investigations of the physiology of the kinin system.

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