Antinociceptive Pharmacology of \(N\)-[[4-(4,5-Dihydro-1H-imidazol-2-yl)phenyl][methyl]-2-[2-[[4-methoxy-2,6-dimethylphenyl] sulfonyl][methylamino]ethoxy]-N-methylacetamide, Fumarate (LF22-0542), a Novel Nonpeptidic Bradykinin B\(_1\) Receptor Antagonist


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ABSTRACT

The antinociceptive pharmacology of \(N\)-[[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl][methyl]-2-[2-[[4-methoxy-2,6-dimethylphenyl] sulfonyl][methylamino]ethoxy]-N-methylacetamide fumarate (LF22-0542), a novel nonpeptidic \(B_1\) antagonist, was characterized. LF22-0542 showed high affinity for human and mouse \(B_1\) receptors with virtually no affinity for the human \(B_2\) receptor; a selectivity index of at least 4000 times was obtained when LF22-0542 was profiled throughout binding or cell biology assays on 64 other G-protein-coupled receptor, 10 ion channels, and seven enzymes. LF22-0542 was a competitive \(B_1\) receptor antagonist and elicited significant antinociceptive actions in the mouse acetic acid-induced writhing assay, as well as in the second phases of formalin-induced nociception in mice and in both the first and second phases of the formalin response in rats. LF22-0542 was active after s.c. but not p.o. administration. In \(B_1\) receptor knockout (KO) mice, acetic acid and formalin responses were significantly reduced and LF22-0542 had no additional effects in these animals. LF22-0542 alleviated thermal hypersensitivity in both acute (carrageenan) and persistent inflammatory (complete Freund’s adjuvant) pain models in rats. LF22-0542 produced a full reversal of experimental neuropathic thermal hypersensitivity but was inactive in reversing nerve injury-induced tactile hypersensitivity in rats. In agreement with this observation, \(B_1\) KO mice subjected to peripheral nerve injury did not show thermal hypersensitivity but developed nerve injury-induced tactile hypersensitivity normally. The data demonstrate the antihyperalgesic actions of a selective systemically administered \(B_1\) receptor antagonist and suggest the utility of this class of agents for the treatment of inflammatory pain states and for some aspects of neuropathic pain.

Pain-relieving therapies for acute and chronic pain currently rely primarily on nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and a diverse group of drugs used as direct or adjunctive treatments (e.g., antidepressants, anticonvulsants, local anesthetics, and \(\alpha_2\)-adrenoreceptor agonists). These types of drugs are limited in their clinical utility as a consequence of significant adverse effects that often limit tolerability (i.e., they possess a relatively low therapeutic index) and prevent “dosing to effect” to achieve adequate or tolerable management of some chronic pain states, such as neuropathic pain.

Kinins are a family of peptides containing nine to 11 amino acids that are released from large inactive precursors called kininogens by enzymatic activity. In mammals, the two main kinins are bradykinin (BK) (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and kallidin (KD) (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (Bhoola et al., 1992). Pharmacological experiments have supported the existence of two receptors (Regoli and Barabe, 1980). Pharmacological experiments have supported the existence of two receptors (Regoli and Barabé, 1980), and molecular

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; BK, bradykinin; KD, kallidin; \(B_1\), bradykinin 1 receptor; \(B_2\), bradykinin 2 receptor; KO, knockout; LF22-0542 \(N\)-[[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl][methyl]-2-[2-[[4-methoxy-2,6-dimethylphenyl] sulfonyl][methylamino]ethoxy]-N-methylacetamide, fumarate; HEK, human embryonic kidney; TES, (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; CHO, Chinese hamster ovary; U46619, 11,19 epoxymethano-prostaglandin H\(_2\).
cloning has led to two distinct G-protein-coupled receptors denoted B₁ and B₂ (Hess et al., 1992; Menke et al., 1994). Both BK and KD are cleaved by carboxypeptidase N which removes the C-terminal Arg residue resulting in biologically active peptides that act as selective agonists for the kinin B₁ receptor (Bhoola et al., 1992; Marceau and Regoli, 2004). The B₂ receptor is constitutively expressed and found in a number of tissues whereas the B₁ receptor is inducible after inflammation (Marceau and Regoli, 2004), suggesting a role in promoting pain. Both receptors couple to the same signal transduction mechanisms including Go₁ and Go₄. Whereas the B₂ receptor undergoes rapid desensitization after agonist activation, the B₁ receptor is not rapidly desensitized, hence allowing for ongoing activation, promoting pain and inflammation (Blaukat et al., 1999). Therefore, compounds that block the B₁ receptor may prove useful in long-term treatment of inflammatory pain states with a lack, or limited, scope of side effects.

Bradykinin promotes polymodal nociceptor activation and hyperalgesia (Dray et al., 1992) can sensitize nociceptors after the release of prostaglandins, cytokines, and nitric oxide and facilitate the release of substance P and calcitonin gene-related peptide from rat sensory neurons in culture (Vasko et al., 1994). Bradykinin is well known to act at B₂ receptors located on primary afferent fibers to promote pain (Juan and Lembec, 1974; Dray, 1997). Numerous studies have demonstrated antinoceptive effects of B₂ antagonists after acute inflammation (Perkins et al., 1993; Rupniak et al., 1997). Recent evidence suggests that B₁ receptors are also constitutively expressed at low levels in the rat (Wotherspoon and Winter, 2000). Peptidic B₁ receptor antagonists have been shown to elicit antinoiception, and antihyperalgesic activity in several models of persistent inflammatory and visceral pain (Perkins et al., 1993; Davis and Perkins, 1994; Khasar et al., 1995; Rupniak et al., 1997; Jaggar et al., 1998; Bélichard et al., 2000; Calixto et al., 2004), but the potential actions of systemically available nonpeptidic B₁ receptor antagonists are relatively unexplored.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), C57BL/6 mice (Elevage Janvier, Laval, France), Swiss mice (Elevage, Janvier), and B₁ receptor wild-type and knockout (−/−) mice were used in these experiments. The B₁ receptor wild-type and knockout (−/−) mice were a generous gift of Dr. Michael Bader (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) and were produced and maintained as described previously (Pesquero et al., 2000). Male Wistar rats and Swiss mice (Elevage Janvier) were also used for isolated organ experiments. All procedures were in accordance with the policies and recommendations of the National Institutes of Health Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the Animal Care and Use Committee of the University of Arizona and the Ethical Committee on animal use at Fournier.

Synthesis

LF22-0542 was discovered through a functional human umbilical vein-based screening designed to identify highly selective and potent antagonists at the B₁ receptor. LF22-0542 was synthesized at Fournier.

In Vitro Studies

Competition Binding Assay of LF22-0542 to the Human or Mouse Recombinant B₁ Receptor. HEK 293 cells stably expressing the human or mouse B₁ receptor were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 1% Glutamax (v/v), 1% nonessential amino acid (v/v), 1 mM sodium pyruvate, 100 mg/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum. HEK 293 cells were rinsed twice with Ca²⁺/Mg²⁺-free ice-cold PBS and scraped from the dishes with a rubber policeman in 5 ml of binding buffer (25 mM 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulfonic acid (TES), 1 mM 1,10-phenanthroline, 140 mg/ml bacitracin, and 0.1% bovine serum albumin at pH 7.4). They were homogenized with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at 40,000g for 20 min. The pellet was then resuspended in binding buffer and stored in liquid nitrogen. Binding experiments were performed at room temperature with a 20- to 40-µg membrane protein/assay in binding buffer. For saturation experiments, [³H]des-Arg¹⁰-kallidin (10.1±0.5 nM) was incubated for 1 hour in a final volume of 500 µl. The assay was terminated by filtration on Whatman GF/B filters presoaked for 2 h in 0.1% polyethyleneimine (w/v). Filters were rinsed three times with 5 ml of ice-cold 50 mM TES, and the radioactivity was determined by liquid scintillation counting in 5 ml of Optima Gold (Packard, Brea, USA). Nonspecific binding was determined in the presence of 10 µM des-Arg¹⁰-kallidin. Competition binding experiments were carried out in the presence of [³H]des-Arg¹⁰-kallidin at a dose equal to the Kᵢ in competition with various concentrations of LF22-0542. All assays were carried out in duplicate. Protein concentration was measured by the method of Bradford (1976).

Competition Binding Assay of LF22-0542 to the Human Recombinant B₂ Receptor. Chinese hamster ovary (CHO) K1 cells expressing the human B₂ receptor (Hess et al., 1992) were maintained in Ham’s F12 medium containing 10% fetal calf serum, 4.5 g/l glucose, 100 mg/ml streptomycin, and 10% fetal bovine serum. CHO cells were rinsed twice with Ca²⁺/Mg²⁺-free ice-cold phosphate-buffered saline and scraped from the dishes with a rubber policeman in 5 ml of binding buffer (25 mM TES, 1 mM 1,10-phenanthroline, 140 mg/ml bacitracin, and 0.1% bovine serum albumin) at pH 6.8. They were homogenized with a Polytron (Kinematica) and centrifuged at 40,000g for 20 min. The pellet was then resuspended in binding buffer and stored in liquid nitrogen. Binding experiments were performed at room temperature with 20 to 40 µg of membrane protein/
Krebs’ solution maintained at 37°C and bubbled with 95% O2 and 5% CO2. The assay was terminated by filtration on Whatman GF/B filters pre-soaked for 2 h in 0.1% polyethyleneimide (w/v). Filters were rinsed three times with 5 ml of ice-cold 50 mM TES, and the radioactivity was determined by liquid scintillation counting in 5 ml of Optima Gold (Packard). Nonspecific binding was determined in the presence of 10 μM bradykinin. Competition binding experiments were carried out in the presence of [3H]bradykinin at a dose equal to the KB, in competition with various concentrations of LF22-0542 for 90 min at room temperature. All assays were carried out in duplicate. Protein concentration was measured by the method of Bradford (1976).

Isolated Organs

Human Umbilical Vein. With the approval of the Ethical Committee of Clinique Ste Marthe (Dijon, France), human umbilical cords were collected postdelivery and immediately placed in Krebs’ solution of the following composition: 119 mM NaCl, 4.7 mM KCl, 1.5 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, 5.5 mM glucose, and 0.026 mM EDTA. Umbilical vein rings (3–4 mm in length) were incubated overnight in 20-ml jacketed organ baths containing Dulbecco’s modified Eagle’s cell culture medium supplemented with 1% fetal calf serum and penicillin (10 IU/ml)-streptomycin (10 μg/ml) maintained at 37°C and bubbled with 95% O2 and 5% CO2 to induce kinin B1 receptor. Then they were set up in 8-ml jacketed organ baths containing Krebs’ solution maintained at 37°C and bubbled with 95% O2 and 5% CO2. Strips were left unstretched for 1 h, during which the bath fluid was changed every 15 min with fresh solution. Strips were then stretched to 1 g. All rings were contracted by KPSS (Krebs’ solution in which NaCl was replaced by KCl) to assess their contractility. After washing twice with normal Krebs’ solution and return to the baseline, captopril (10 μM), thiorphan (1 μM), mepyramine (1 μM), and indomethacin (3 μM), were added into the organ bath. Thirty minutes later vehicle or LF22-0542 was injected in the bath; this mixture was incubated for 30 min, and concentration-response curves to des-Arg10-kallidin were obtained. Each segment was used for a single concentration-response curve. The contractile responses to des-Arg10-kallidin were expressed as a percentage of the maximal contraction to carbachol.

Data Analysis. Binding competition data and concentration-response curves for BK- or des-Arg10-kallidin-induced contractions were analyzed using GraphPAD InPlot (GraphPAD Software, San Diego, CA). The maximal binding at equilibrium (Bmax) and the equilibrium dissociation constant (KB) were derived from saturation curves fitted with a one-site ligand binding model. Values of inhibitory binding constants (Ki) were obtained from the Cheng-Prusoff equation: $K_i = [\text{IC}_{50}/1 + [I]/K_B$ where L and KB are the concentration and equilibrium dissociation constants of the radioligand, respectively, and IC50 is the concentration of competing ligand reducing specific binding by 50%. EC50 was the concentration of agonist needed to reach 50% of the maximal response and was calculated using least-square analysis (Tallarida and Murray, 1981). The pA2 value ($-\log K_B$) was obtained according to the following equation: $K_B = [B]/(\text{concentration ratio} - 1)$ where [B] is the concentration of the antagonist, and the concentration ratio is the EC50 in the presence of the antagonist divided by the EC50 in the absence of antagonist. Schil analysis was used to calculate pA2 values when the Schid slope plot did not differ from unity and when maximal contractions to des-Arg10-kallidin were not significantly affected whatever the concentration of LF22-0542. Statistical analysis of the Schid slope was performed using the Student’s t test for slope.

In Vivo Studies

Injections. For i.v. administration, animals were placed in restrainers, and a disposable 1-ml syringe equipped with a 30-gauge disposable needle was used for injections. The needle was inserted into the tail vein at a 25° angle, and a small amount of blood was drawn back into the syringe before injection of either compounds or vehicle to assure injection into the vein. Injection of compounds or vehicle was performed over a 5-s period for consistency purposes. After injection the needle was removed from the tail vein and gentle pressure was applied at the site of injection to prevent loss of fluid from the site of injection. Subcutaneous injections were performed by manually holding the animal and inserting a 30-gauge disposable needle on a disposable 1-ml syringe into the abdominal region of the animal, assuring that the needle remained between the muscle and the skin of the animal. Injections of compounds were performed over a 5-s period and were noted as positive by the development of an out-pocketing of the skin at the site of injection. Oral delivery was accomplished by using an 18-gauge gavage needle attached to a 10-ml syringe.

Intrathecal Catheterization and Injections. While under halothane anesthesia, some groups of rats were implanted with intrathecal catheters (PE-10, 7.8 cm) for drug administration at the level of the lumbar spinal cord. Animals were allowed to recover for 7 days. On the test day, compounds or vehicle was administered in a 5-μl volume using a 10-μl Hamilton syringe followed by a 1-μl air bubble and a 9-μl saline push. The 1-μl air bubble is observed during injection to assure movement of compound or vehicle through the intrathecal catheter. Injections were performed over a 5- to 10-s period to prevent rapid bolus irritation to the lumbar region as well as to prevent backflow via the intrathecal catheter.

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Acute Nociceptive Assays

Des-Arg<sup>9</sup>-Bradykinin-Induced Paw Rising in Mice. After a 30-min acclimation period in individual Plexiglas observation boxes, mice received an intraplantar injection (20 μl) of the B<sub>1</sub> receptor agonist des-Arg<sup>9</sup>-bradykinin (300 nmol) into one hindpaw. The duration of leg raising and licking of the injected paw was recorded immediately and continuously thereafter for 15 min. LF22-0542 (0.001, 0.01, 0.1, and 1 mg/kg) or vehicle (saline) was given intravenously 10 min before the injection of des-Arg<sup>9</sup>-bradykinin. Percentage inhibition was calculated as follows: ([Dv - DT]/Dv × 100), where Dv is the duration of leg rising and licking for the vehicle group and DT is the duration of leg rising and licking for the treated group.

Acetic Acid-Induced Writhing Assay of Visceral Pain in Mice. Mice were habituated to individual observation boxes and received an injection of 0.1 ml of acetic acid (0.6%) in the abdominal cavity to evoke writhing. The number of writhes was recorded continuously immediately after the injection of acetic acid for 20 min. LF22-0542 (10 mg/kg) or vehicle (saline) was injected s.c. 30 min before the injection of acetic acid.

Tail-Flick Test in Mice. The hot-water tail-flick test was performed by dipping the distal third of the tail into a water bath maintained at either 48, 52, or 55°C. The latency until a rapid flick of the tail or tail withdrawal from the bath was determined and compared. A 10-s cutoff was used to avoid tissue damage. Data were converted to percentage antinociception by the formula (response latency - baseline latency)/cutoff - baseline latency × 100, and the resulting values were used to generate dose-response curves.

Hot-Plate Test in Mice and Rats. The hot-plate test was performed by placing mice individually on a hot plate maintained at either 48°C or 52°C with a Plexiglas enclosure of 30 × 30 × 30 cm. The latency to hindpaw lick or jump from the hot-plate was determined. A 60-s cutoff was used to avoid tissue damage. Rats were placed individually on a hot-plate maintained at 52°C with a Plexiglas enclosure of 30 × 30 × 30 cm. The latency to hindpaw lick or jump from the hot-plate was determined. A 60-s cutoff was used to avoid tissue damage. Data were converted to percentage antinociception by the formula, (response latency - baseline latency)/cutoff - baseline latency × 100, and the resulting values were used to generate dose-response curves.

Inflammatory Pain Assays

Formalin-Induced Nociception in Mice and Rats. Mice were habituated to individual observation boxes and received an intraplantar injection (20 μl) of formalin (0.92%) into one hindpaw. The duration of leg raising and licking of the injected paw were recorded immediately and continuously thereafter for 30 min. The nociceptive response was divided into two phases, the early one between 0 and 5 min and the late one between 15 and 30 min. LF22-0542 (10 mg/kg) or vehicle (saline) was given s.c. 30 min before the injection of formalin. The procedure was modified for rats. Animals were habituated for 30 min to the environment in a clear 30 × 30 × 30 cm chamber with mirrors placed at a 45° angle under the chambers to give an unobstructed view of the paws. Animals were restrained manually, and 2% formalin (made fresh the day of the experiment) was injected s.c. (50 μl) into the plantar surface of the left hindpaw with a 30-gauge needle. The number of paw flinches was then recorded in 5-min intervals from the time of injection for a 60-min period. Elevations of the paw and licking and biting of the injected paw were counted as “flinches”. The acute or first phase of the nociceptive response peaked at 5 min after formalin injection and the inflammatory or second phase peaked at 30 min after formalin injection. The number of flinches in the first 10 min was representative of the acute phase, and the number of flinches from 10 until 60 min was representative of the second phase. LF22-0542 or vehicle was administered by the i.v. route 5 min before formalin injection. Antinociception for either phase I or phase II was calculated as follows: percentage antinociception = 100 [number of flinches in control animal - number of flinches in drug-treated animal]/number of flinches in control animal). Potency (A<sub>50</sub>) is calculated from the full dose-response curve for both phase I and phase II.

Carrageenan-Induced Acute Inflammatory Pain. To test the antinociceptive activity of LF22-0542 under acute inflammatory conditions, we measured the latency to paw withdrawal from a noxious thermal stimulus before and 3 h after injection of a 50-μl solution of 2% carrageenan into the plantar surface of the hindpaw. Animals were placed in 3 × 3 cm Plexiglas boxes on top of a glass plate that was maintained at 30°C and allowed to habituate for a period of 45 min. For baseline paw withdrawal latencies, a heat source that increased in intensity from a non-noxious temperature to a noxious temperature (within 1 to 16 s in naive animals) from under the glass plate was directed onto the plantar surface of the right hindpaw with the focus of the light beam no larger than a 3- to 5-mm diameter. The time to withdrawal of the hindpaw from the heat source was recorded. A maximum cutoff of 40 s was used to prevent tissue damage as the intensity of the heat source continued to increase. The post-carrageenan baseline was reestablished 3 h after the carrageenan injections and only animals with a significant decrease in the latency of hindpaw withdrawal from the thermal stimulus (thermal hypersensitivity) were further tested. Animals were administered LF22-0542 or vehicle, and hindpaw withdrawal latencies were tested at 15-min intervals over a 60-min period. Antihyperalgesia (thermal hypersensitivity) and antinociception were calculated as follows: percentage activity = 100 [(test paw withdrawal latency - post-carrageenan baseline paw withdrawal latency)/(precarrageenan baseline paw withdrawal latency - post-carrageenan baseline paw withdrawal latency)].

CFA-Induced Persistent Inflammatory Pain. The latency of the animal to withdrawal of the hindpaw from a noxious thermal stimulus was determined as described above. 3 days after the injection of the hindpaw with a 50-μl solution of CFA. Animals were placed in 3 × 3-cm Plexiglas boxes on top of a glass plate that was maintained at 30°C. Three days after CFA, animals were allowed to habituate for a period of 45 min and were the baseline was reestablished (post-CFA baseline). Only animals with a significant thermal hypersensitivity, demonstrated by a decrease in the latency of paw withdrawal from the pre-CFA baseline, were tested further. Animals were administered LF22-0542 or vehicle, and paw withdrawal latencies were tested at 15-min intervals over a 60-min period. Antinociception or antihyperalgesia (thermal hypersensitivity) was calculated as follows: percentage activity = 100 [(test paw withdrawal latency - post-CFA baseline paw withdrawal latency)/(precarrageenan baseline paw withdrawal latency - post-CFA baseline paw withdrawal latency)].

Experimental Nerve Injury (Sciatic Nerve Ligation). Nerve ligation injury produces signs of neuropathic dysesthesias, including tactile allodynia, thermal hypersensitivity, and guarding of the affected paw. Rats or mice were anesthetized with 2% halothane in O₂ delivered at 2 l/min. The skin over the caudal lumbar region was incised, and the muscles were retracted. The L₉ and L₈ spinal nerves were exposed, carefully isolated, and tightly ligated with a 4-0 silk suture distal to the dorsal root ganglion. After homeostatic stability was assured, the wounds were sutured, and the animals were allowed to recover in individual cages. Sham-operated animals were prepared in an identical fashion except that the L₉/L₈ spinal nerves were not ligated. Any animals exhibiting signs of motor deficiency were euthanized.

Sensory Thresholds to Thermal Stimulation of the Paw. The method of applying a thermal heat source to the hindpaw of the animal was used to assess paw withdrawal latency to a thermal nociceptive stimulus using a standard apparatus (Plantar Analgesia Instrument; Stoelting Co, Wood Dale, IL). Animals were placed in 3 × 3-cm Plexiglas boxes on top of a glass plate that was maintained at 30°C. Animals were allowed to habituate for a period of 45 min. For baseline paw withdrawal latencies, a heat source that increased in intensity from a non-noxious temperature to a noxious tempera-
ture (within 1 to 16 s in naive animals) from under the glass plate was directed onto the plantar surface of the right hindpaw with the focus of the light beam being no larger than a 3- to 5-mm diameter and the time to withdrawal the paw from the heat source was recorded. A maximum cutoff of 40 s was used to prevent tissue damage. Animals were administered LF22-0542 or vehicle, and paw withdrawal latencies were tested at 15-min intervals over a 60-min period. Antinociception, or antihyperalgesia (thermal hypersensitivity, was calculated as follows: percentage activity = 100 ([test paw withdrawal latency – postsurgery baseline paw withdrawal latency]/[presurgery baseline paw withdrawal latency – postsurgery baseline paw withdrawal latency]).

Sensory Thresholds to Non-Noxious Tactile Stimuli in Rats and Mice. The paw withdrawal thresholds to tactile stimuli were determined in response to probing with calibrated von Frey filaments. The animals were kept in suspended cages with wire mesh floors and the von Frey filaments were applied perpendicularly to the plantar surface of the paw of the animal until it buckled slightly and were held for 3 to 6 s. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the nonparametric method of Dixon (1980). An initial probe equivalent to 2.00 g was applied, and if the response was negative the stimulus was increased one increment; otherwise a positive response resulted in a decrease of one increment. The stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was observed. This “up-down” method was repeated until three changes in behavior were determined. The pattern of positive and negative responses was tabulated. The 50% paw withdrawal threshold is determined as (10 (Xf + k))/10,000, where Xf = the value of the last von Frey filament used, k = Dixon value for the positive/negative pattern, and M = the mean (log) difference between stimuli. Data were converted to percentage “antiallodynia” by the formula: percentage activity = 100 × (test value – control value)/(15 g – control value). Only animals that demonstrate tactile hypersensitivity were used in all studies. Animals were administered LF22-0542 or vehicle and paw withdrawal latencies were tested at 15-min intervals over a 60-min interval.

Motor Coordination Assays

Rotarod Test in Mice. Potential central nervous system effects, including sedation or nonspecific motor effects, were evaluated by measuring the ability of mice to maintain balance on an accelerating rotating rod (rate of rotation was 4 to 40 rpm over a 5-min period) after s.c. administration of LF22-0542 (10, 30, and 100 mg/kg) or vehicle. Mice were conditioned before the experiment (at least eight trials), and animals were selected as those remaining on the Rotarod for 300 s. On the day of experiment, three trials were consecutively performed to establish baseline values. Mice were tested at 30, 60, 90, and 120 min after administration.

Spontaneous Motor Activity in Rats. Rats were habituated in cages equipped with photocells for 30 min and at the end of the habituation period were injected s.c. with LF22-0542 (10, 30, and 100 mg/kg) or the corresponding vehicle and placed in their home cages. Thirty minutes later, rats were placed with no adaptation in the activity cages equipped with photocells to measure horizontal motility, which was recorded for 60 min.

Statistics

Data were analyzed by analysis of variance and, where significance was indicated, by Student’s t test for grouped data. The significance criterion was p < 0.05 throughout. Results are reported as the mean activity score and S.E.M. Statistics were calculated using a proprietary software program (FlashCalc; Momentum Engineering Software, Inc., Tucson, AZ) for all in vivo studies.

Results

Characterization of LF22-0542 in Vitro

LF22-0542 (Fig. 1A) competed with [3H]des-Arg10-kallidin binding to the cloned human and mouse B1 receptor with K_i values of 0.35 and 6.5 nM, respectively (Fig. 1B). LF22-0542 had no affinity for the human mouse and rat B2 receptor up to concentrations exceeding 10 μM (data not shown), and a selectivity index of at least 4000 × was obtained when LF22-0542 (5 μM) was profiled throughout binding or cell biology assays on 64 other GPCRs, 10 ion channels, and seven enzymes. LF22-0542 alone did not produce contraction of the human umbilical vein but elicited a concentration-related antagonism of contractions induced by des-Arg10-kallidin with no decrease in the maximal response, suggesting competitive antagonism (Fig. 1C).

Acute Nociceptive Assays. Application of des-Arg9-bradykinin elicited a significant degree of paw rising in WT but not in bradykinin B1 receptor KO mice (Fig. 2A). In addition, although LF22-0542 (10 mg/kg s.c., 10 min before des-Arg3-bradykinin) inhibited the des-Arg3-bradykinin-induced response in WT mice, it had no effect in B1 KO mice (Table 1). In C57BL/6 mice, des-Arg9-bradykinin produced significant leg rising. The baselines of duration of leg rising and licking in vehicle and des-Arg3-BK-treated control mice were 8.0 ± 3.3 and 66.4 ± 9.9 s, respectively, and these responses were...
significantly increased the latency to hindpaw lick or jump compared with vehicle-treated animals from $14.1 \pm 1.1$ to $21.0 \pm 3.2$ s ($p < 0.05$) and from $14.7 \pm 1.2$ to $21.7 \pm 2.2$ s ($p < 0.05$) at 30 and 60 min postadministration, respectively.

Evaluation of WT and B1 KO mice in the tail-flick assay showed a significant hypoalgesia of B1 KO mice at the highest stimulus intensities. A small, but significant, increase in the tail-flick latencies was detected in B1 KO mice at temperatures of $52^\circ$C ($7.1 \pm 0.6$ s) and $55^\circ$C ($5.4 \pm 0.6$ s) compared with the WT mice at $52^\circ$C ($3.2 \pm 0.4$ s) and $55^\circ$C ($2.1 \pm 0.3$ s) (Fig. 3A). In the hot-plate assay, B1 KO mice were hypoalgesic compared with B1 WT mice. At $52^\circ$C, latency to hindpaw lick or jump was significantly greater in B1 KO than in WT mice ($33.7 \pm 4.0$ s versus $13.0 \pm 1.3$ s) (Fig. 3B). In contrast, there were no significant differences between B1 KO ($13.3 \pm 1.0$ s) and WT mice ($11.8 \pm 0.9$ s) in baseline paw withdrawal latencies using the radiant heat paw withdrawal assay, and responses to von Frey filaments were not different ($1.6 \pm 0.2$ and $1.5 \pm 0.1$ g, in WT and B1 KO mice, respectively) (Fig. 3, C and D).

**Formalin Flinching in Rats.** Injection of a 2% formalin preparation into the plantar surface of the hindpaw resulted in 74.5 $\pm$ 6.4 paw flinches during the first 10 min, representing the first phase of the response (Fig. 4, A and B). This was followed by a quiescent period of approximately 5 min and a subsequent second phase of flinching with a peak number of flinches 45 min after formalin administration at 99.6 $\pm$ 17.1 flinches (Fig. 4, A and C). Intravenous administration of

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>B1 WT</th>
<th>B1 KO</th>
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<tbody>
<tr>
<td>Saline</td>
<td>8.0 $\pm$ 3.3</td>
<td>10.3 $\pm$ 4.4</td>
</tr>
<tr>
<td>Des-Arg^9-bradykinin + vehicle</td>
<td>66.4 $\pm$ 9.9</td>
<td>11.0 $\pm$ 4.9</td>
</tr>
<tr>
<td>Des-Arg^9-bradykinin + LF22-0542 (10 mg/kg s.c.)</td>
<td>15.3 $\pm$ 2.9</td>
<td>9.3 $\pm$ 3.5</td>
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</tbody>
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blocked in a dose-dependent manner by i.v. administration of LF22-0542 (Fig. 2B). Administration of formalin elicited significantly nocifensive responses in the second phase in WT mice. This response was significantly inhibited by LF22-0542 (Fig. 2C). LF22-0542 had no effect in the first phase of the formalin response. The formalin response was significantly reduced in B1 KO mice, and LF22-0542 produced no effect on the response in these animals (Fig. 2C). Administration of acetic acid elicited a significant number of writhing responses in WT mice, and this effect was significantly antagonized by LF22-0542 (Fig. 2D). The number of writhes diminished in B1 KO mice, and LF22-0542 had no effect in these animals (Fig. 2D). In male Sprague-Dawley rats for the hot-plate test performed at $52^\circ$C, LF22-0542 (10 mg/kg s.c.)
The calculated response (Fig. 4). The calculated reduction of both the first and the second phases of LF22-0542 5 min before formalin was inactive (data not shown). Administration of s.c. LF22-0542 30 min before formalin showed a dose-related inhibition in both the phase I and phase II responses (data not shown). At a dose of 1 mg/kg s.c., LF22-0542 produced 39.6 ± 10.3 and 44.9 ± 9.8% inhibition of the phase I and II flinching responses, respectively. At 10 mg/kg s.c. LF22-0542 produced 51.7 ± 8.5 and 45.7 ± 3.1% inhibition of the phase I and II flinching responses, respectively. LF22-0542 given orally at a dose of 0.5 mg/kg 30 min before formalin was inactive (data not shown).

**Carrageenan-Induced Acute Inflammatory Pain in Rats.** Administration of carrageenan produced a significant decrease in paw withdrawal latencies when evaluated at the 3-h time point. Paw withdrawal latencies were significantly diminished from 20.6 ± 0.5 s in naive animals to 10.8 ± 0.8 s, demonstrating the expected inflammation-induced thermal hypersensitivity. The i.v. administration of LF22-0542 significantly reversed the carrageenan-induced reduction in paw withdrawal latencies to control levels in a dose- and time-related fashion. The peak effect of LF22-0542 was seen 15 min after administration (Fig. 5). The effect diminished with time and a return to postcarrageenan baselines was observed by 60 min after LF22-0542. Administration of LF22-0542 by the s.c. route showed similar antihyperalgesic activity with a dose of 3 mg/kg resulting in a 57.4 ± 19.9% response at 30 min after administration with a complete return to postcarrageenan paw withdrawal latencies by 60 min (data not shown).

**Complete Freund’s Adjuvant-Induced Persistent Inflammatory Pain in Rats.** The hindpaw injection of CFA produced a significant increase in the sensitivity of the hindpaw to thermal stimuli when evaluated 3 days after administration. Baseline withdrawal latencies were 20.1 ± 0.6 s before CFA and were reduced to 10.7 ± 0.7 s, indicating significant thermal hypersensitivity. Intravenous administration of LF22-0542 produced a dose- and time-related reversal of CFA-induced thermal hypersensitivity with an A50 of 0.97 mg/kg (CI 0.38–2.52) at the 30-min time point with return to baseline by 60 min after drug administration. LF22-0542 was also active at a dose of 10 mg/kg s.c.; this dose resulted in 61.5 ± 18.3% antihyperalgesic effect at the 30-min time point with return to predrug baselines by 60 min.

**Spinal Nerve Ligation-Induced Neuropathic Pain in Rats.** Sensory thresholds to either a thermal stimulus or non-noxious probing with von Frey filaments were not altered by sham SNL surgery. LF22-0542 had no effect on responses to heat or tactile stimuli in sham-operated rats. As expected, SNL produced significant thermal hypersensitivity and tactile hypersensitivity. Paw withdrawal latencies elicited by a thermal stimulus were significantly reduced to 12.2 ± 0.7 s from a presurgery baseline of 22.2 ± 0.9 s 7 days after SNL. Intravenous LF22-0542 produced a significant dose-related reversal of thermal hypersensitivity with an A50 of 1.0 mg/kg (CI 0.28–1.78) at the 30-min time point (Fig. 6A). Likewise, s.c. administration of LF22-0542 7 days after SNL resulted in significant dose- and time-related reversal of thermal hypersensitivity with an A50 of 2.8 mg/kg (CI 1.0–7.4) at the 30-min time point (data not shown). In contrast, no significant effects were seen against tactile hypersensitivity with either i.v. or s.c. LF22-0542. Baseline mechanical thresholds were measured at 15.00 ± 0.0 g with a significant decrease measured 7 days after SNL to thresholds of 2.6 ± 0.2 g. LF22-0542, when administered either by the i.v. (1, 3, or 10 mg/kg) (Fig. 6B) or s.c. (3, 10, or 30 mg/kg, data not shown) routes resulted in no difference from postsurgery baseline thresholds. No significant activity of LF22-0542 was observed after intrathecal administration against either SNL-induced thermal or tactile hypersensitivity up to doses of 100 μg over a period of 60 min (data not shown).

**Spinal Nerve Ligation-Induced Neuropathic Pain in Mice.** Sham SNL surgery did not alter sensory thresholds to either a thermal stimulus or non-noxious tactile stimuli in either WT or B1 KO mice (Fig. 7, A and B). Significant mechanical hypersensitivity was seen on day 3 after nerve injury in both the WT (0.2 ± 0.1 g) and B1 KO mice (0.2 ± 0.1 g) compared with sham nerve-injured WT (1.4 ± 0.3 g).
and B1 KO (1.7 ± 0.3 g) mice (Fig. 7A). The mechanical hypersensitivity lasted for 9 days with no signs of attenuation in either WT or B1 KO mice. SNL also produced significant thermal hypersensitivity in WT (7.1 ± 1.2 s) mice compared with sham nerve-injured WT (2.1 ± 0.3 s) mice (Fig. 7B). In contrast, B1 KO mice showed a baseline of 14.1 ± 2.1 s, which was not different after SNL injury. The SNL-induced thermal hypersensitivity seen in WT mice lasted for 9 days with no signs of attenuation.

**Rotarod Test and Spontaneous Motor Activity.** LF22-0542 at 10 and 30 mg/kg s.c. had no effect on Rotarod performance of mice. However, at 100 mg/kg s.c., LF22-0542 transiently reduced the time spent on the Rotarod to 47% of baseline (Fig. 8A). In rats, LF22-0542 up to 100 mg/kg did not significantly change horizontal motility over 60 min after treatment compared with vehicle-treated animals, although there was a significant decrease in locomotor activity at the 0- to 10-min period at the highest dose (Fig. 8B).

**Discussion**

The availability of LF22-0542, a novel B1 receptor antagonist with subnanomolar affinity for the human B1 receptor, at least 4000-fold selectivity for B1 versus B2 receptors, and exceptional selectivity compared with other GPCRs, enzymes, and ion channels has allowed an evaluation of the role of the B1 receptor in pain states. LF22-0542 is a competitive, high-affinity antagonist at the B1 receptor in vitro. Blockade of the B1 receptor with LF22-0542 elicited significant antinoceptive and antihyperalgesic actions in acute, visceral, inflammatory and in some aspects of experimental neuropathic pain. These data were generally in accordance with the effects seen in our laboratories with B1 KO mice, supporting the selectivity of LF22-0542 for the B1 receptor and the potential development of antagonists at this site for clinical treatment of pain states.

Bradykinin has been implicated in multiple inflammatory pain states (Regoli and Barabé, 1980; Bathon and Proud, 1991). Tissue damage results in enhanced levels of des-Arg9-bradykinin, the preferential agonist for the B1 receptor (De-carie et al., 1996) and the B1 receptor itself is up-regulated and overexpressed after injury (Schanstra et al., 1998) and may not show desensitization (Mathis et al., 1996; Faussner et al., 1998).

Peptidic B1 receptor agonists do not affect basal nociceptive thresholds in naive rats (Dray, 1997) suggesting a limited role for the B1 receptor in acute nociceptive processes (Dray et al., 1992; Andreeva and Rang, 1993). In support of this conclusion, peptidic B1 receptor agonists do not result in a change in baseline thresholds to mechanical or thermal stimuli in naive animals (Haley et al., 1989; Dray et al., 1992; Davis and Perkins, 1994) possibly due to the low constitutive level of B1 receptors or nonstable peptidic agonists used in these studies. On the other hand, a potential role of the B1 receptor is supported by observations that B1 receptor knockout mice demonstrate increased baseline thresholds to chem-
that this B₁ antagonist is most active against mild thermal nociceptive stimuli. LF22-0542 was also active in phase I of the formalin test in rats (but not in phase I of the formalin test in mice) at the doses tested. LF22-0542 produced a significant inhibition of acetic acid writhing in B₁ WT mice with reduced responses in B₁ KO mice without further effects of the compound. Collectively, these findings extend the in vitro characterization of LF22-0542 to the whole animal and support the conclusion of high potency and selectivity for the B₁ receptor as well as supporting a role for the B₁ receptor in acute nociceptive processes associated with chemical stimuli and with mild noxious thermal stimuli.

In contrast to studies of acute nociception, numerous studies have shown that B₁ antagonists are antihyperalgesic in animal models of persistent inflammation (Perkins et al., 1993; Bélichard et al., 2000; Rawlingson et al., 2001), as well as in persistent visceral pain (Jaggar et al., 1998). Des-Arg⁹-[Leu⁸]-BK, a B₁ receptor antagonist (Marceau and Regoli, 2004) inhibits carrageenan-induced hyperalgesia and formalin-induced paw flick (Rupniak et al., 1997), as well as edema and hyperalgesia associated with burn injuries (Perkins et al., 1993; Rawlingson et al., 2001). LF22-0542 also showed activity in models of inflammatory pain including significant anti-hyperalgesic actions in the second phase of the formalin response in both mice and rats and in both acute (carrageenan) and persistent (CFA) inflammatory pain. Intravenous LF22-0542 was approximately 5-fold more potent against acute carrageenan-induced inflammatory hyper-sensitivity than CFA-induced hypersensitivity measured 3 days after the inflammatory stimulus, suggesting that the activity of this compound did not depend on up-regulation of the B₁ receptor in these pain states.

Recent findings suggest the requirement for the B₁ receptor in neuropathic pain resulting from injuries to peripheral nerves (Ferreira et al., 2005). The data with LF22-0542 support a significant role of the B₁ receptor in mediating nerve injury-induced thermal hyper-sensitivity but not tactile hyper-sensitivity. As constitutive expression of the B₁ receptor has been reported, although at low levels (Wotherspoon and Winter, 2000), the possible spinal actions of LF22-0542 were evaluated against tactile hypersensitivity; the compound was inactive after intrathecal administration. Modality-specific activity of B₁ antagonists in neuropathic states is also supported by observations in B₁ receptor WT and KO mice. B₁ WT mice showed expected tactile and thermal hypersensitivity. However, B₁ KO animals did not develop thermal hyper-sensitivity but nevertheless showed SNL-induced tactile hypersensitivity that did not differ from the WT animals. Pesquero and colleagues have reported that B₁ KO mice did not develop either tactile or thermal hypersensitivity after nerve injury (Ferreira et al., 2005). In those studies the authors used mice from the same source as in the present studies but used a different model nerve injury (i.e., partial ligation of the sciatic nerve). Although the precise reasons for the discrepancy in the role of B₁ receptors against tactile allodynia are unknown, it may be possible that the two models, SNL and partial sciatic nerve ligation, depend to different degrees on local inflammatory processes and associated roles of immune cells. B₁ receptor mRNA is increased in the ipsilateral lumbar dorsal root ganglia of nerve-injured animals (Lévy and Zochodne, 2000) and des-Arg⁹-[Leu⁸]-BK significantly attenuated nerve injury-induced thermal hyper-

**Fig. 8.** LF22-0542 was tested for motor activity using the Rotarod assay and cages equipped with photocells to monitor locomotion. A, results with LF22-0542 at doses of 10 and 30 mg/kg s.c. were not different from those for vehicle-treated mice over a 2-h test period. The dose of 100 mg/kg s.c. resulted in a reduction of time spent on the Rotarod (i.e., 47% of the time demonstrated by vehicle-treated animals) only at the 30-min time point.

B, there was no significant difference in the ability of LF22-0542 to modulate the locomotion of rats at doses of 10, 30, and 100 mg/kg over a 60-min period (p < 0.05) compared with vehicle-treated rats (n = 8–10 rats at each dose).

The data obtained with the B₁ knockout mice are in agreement with the consequences of systemic B₁ receptor blockade. LF22-0542 produced inhibition of the des-Arg⁹-bradykinin-induced paw raising response in B₁ WT mice, suggesting an appropriate “on target” action of the antagonist. This des-Arg⁹-bradykinin-induced response was diminished in B₁ KO mice (data not shown), and LF22-0542 did not produce further effects in these animals. LF22-0542 showed significant antinociceptive action in the hot-plate test. We note however, that LF22-0542 did not raise the thermal nociceptive threshold above baseline in the sham SNL rats, suggesting a possible difference in stimulus intensity in the two tests. In addition, no difference was observed in the paw withdrawal test in B₁ WT and KO mice. These data suggest
sensitivity (Levy and Zochodne, 2000) in agreement with the present results. SSR240612, a nonpeptidic B1 antagonist, significantly attenuated chronic constriction injury-induced thermal hypersensitivity (Gougat et al., 2004). Thus, whereas the activity of B1 receptor blockade or deletion on nerve injury-induced thermal hypersensitivity is strongly supported, the possible involvement of the B1 receptor in nerve injury-induced tactile hypersensitivity requires additional study. Nevertheless, it is noted that human neuropathic pain is often characterized by spontaneous burning pain and, in a subset of patients, by touch-evoked allodynia (Backonja and Stacey, 2004). These characteristics of the human neuropathic state may reflect different mechanisms and involvement of fiber types with spontaneous burning pain probably being mediated by C-fiber activity with touch-evoked allodynia being mediated by large diameter fibers (Ossipov et al., 2000). The activity of B1 receptor antagonists in animal models of heat evoked hypersensitivity after either inflammatory or experimental neuropathic lesions may suggest possible actions in some, but not all, aspects of clinical states of neuropathy.

The activity profile of this B1 antagonist can thus be summarized as showing antinociception against acute chemical stimuli, and mild activity against noxious thermal stimuli. LF22-0542 was most effective in eliciting antihyperalgesic actions against thermal hypersensitivity resulting from nerve injury or inflammation, although no activity of this compound was observed against tactile allodynia associated with nerve injury, in agreement with results from the B1 KO animals, a finding supporting mechanistic differences between tactile and thermal stimuli after nerve injury (Ossipov et al., 2000). The activity profile of LF22-0542 thus compares well with that of NSAIDs, particularly in light of the limited or lack of antinociceptive activity of NSAIDs in animal models of noxious thermal stimuli (Seguin et al., 1995) and lack of activity in nerve injury-induced thermal and mechanical hypersensitivity (Ossipov et al., 2005). In contrast, opioids have been demonstrated as strong antinociceptive agents in noxious thermal, mechanical, and chemical tests (e.g., Seguin et al., 1995) and are also active in models of experimental neuropathic pain in rodents (Bian et al., 1995) and in human neuropathic pain (Rowbotham et al., 2003).

LF22-0542 was systemically but not orally active (at least at this single dose and time and in this single species) and did not produce significant sedative or motor effects as evaluated by visual inspection or the Rotarod assay and showed minimal, but significant, effects in the initial 10 min of evaluation in horizontal motor activity. Current compounds available for the treatment of neuropathic pain are characterized by significant side effects including mental clouding and sedation, which prevents “dosing to effect” and results in inadequate pain relief in many patients. A comparison of the separation between antihyperalgesic activity and motor side effects suggests that the result with LF22-0542 is at least as favorable as that seen with NSAIDs and better than that seen with opioids (Seguin et al., 1995). In our studies, the dose of LF22-0542 producing an approximate 50% effect against nerve injury- or inflammatory-induced thermal stimuli was approximately 3 mg/kg s.c., and this compound showed an approximate 50% response in the second phase of the formalin response at approximately 1 mg/kg s.c. (i.e., 45% inhibition in phase II). As such, the potency difference to the Rotarod assay would range between 10- and 30-fold, a value that compares well with the potency difference for ibuprofen in late-phase formalin versus Rotarod assay of >26 (Seguin et al., 1995). As expected, opioids show a lower degree of separation between antihyperalgesic actions and motor side effects (Seguin et al., 1995). Equally important, we note that other side effects are likely to be associated with both opiates and NSAIDs, including well-recognized significant effects and/or toxicity on the gastrointestinal tract. In summary, our data with LF22-0542 show that this compound is a highly selective, systemically active nonpeptidic B1 receptor antagonist with potent in vitro and in vivo activity. More importantly, the potency activity of LF22-0542 in animal models of visceral, inflammatory, and in some aspects of neuropathic hypersensitivity suggest that B1 receptors may be exploited therapeutically and that molecules of this class can be useful in the treatment of a broad spectrum of clinical pain conditions.

References


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**Antinociception of a Nonpeptidic B₂ Receptor Antagonist**

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