Pharmacological, Pharmacokinetic, and Primate Analgesic Efficacy Profile of the Novel Bradykinin B₁ Receptor Antagonist ELN441958

Jon E. Hawkinson, Balazs G. Szoke, Albert W. Garofalo, Dennis S. Hom, Hongbing Zhang, Mark Dreyer, Juri Y. Fukuda, Linda Chen, Bhushan Samant, Stellanie Simmonds,1 Karla P. Zeitz, Angie Wadsworth, Anna Liao, Raymond A. Chavez,2 Wes Zmolek, Lany Ruslim, Michael P. Bova, Ryan Holcomb,3 Eduardo R. Butelman, Mei-Chuan Ko, and Annika B. Malmberg4


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ABSTRACT

The bradykinin B₁ receptor plays a critical role in chronic pain and inflammation, although efforts to demonstrate efficacy of receptor antagonists have been hampered by species-dependent potency differences, metabolic instability, and low oral exposure of current agents. The pharmacology, pharmacokinetics, and analogical efficacy of the novel benzamide B₁ receptor antagonist 7-chloro-2-[3-[(9-pyrindin-4-yl)-3,9-diazaspiro[5.5]undecanecarbonyl]phenyl]-2,3-dihydro-isoindol-1-one (ELN441958) is described. ELN441958 competitively inhibited the binding of the B₁ agonist ligand [³H]desArg¹⁰-gly-Pro²-Arg⁹-bradykinin (DAKD) to IMR-90 human fibroblast membranes with high affinity (Ki = 0.26 ± 0.02 nM). ELN441958 potently antagonized DAKD (but not bradykinin)-induced calcium mobilization in IMR-90 cells, indicating that it is highly selective for B₁ over B₂ receptors. Antagonism of agonist-induced calcium responses at B₁ receptors from different species indicated that ELN441958 is selective for primate over rodent B₁ receptors with a rank order potency (Ki, nanomolar) of human (0.12 ± 0.02) ~ rhesus monkey (0.24 ± 0.01) > rat (1.5 ± 0.4) > mouse (14 ± 4). ELN441958 had good permeability and metabolic stability in vitro consistent with high oral exposure and moderate plasma half-lives in rats and rhesus monkeys. Because ELN441958 is up to 120-fold more potent at primate than at rodent B₁ receptors, it was evaluated in a primate pain model. ELN441958 dose-dependently reduced carrageenan-induced thermal hyperalgesia in a rhesus monkey tail-withdrawal model, with an ED₅₀ ~3 mg/kg s.c. Naltrexone had no effect on the antihyperalgesia produced by ELN441958, indicating a lack of involvement of opioid receptors. ELN441958 is a novel small molecule bradykinin B₁ receptor antagonist exhibiting high oral bioavailability and potent systemic efficacy in rhesus monkey inflammatory pain.

Antagonism of bradykinin receptors may prove to be a viable therapeutic strategy to reduce pain and inflammation. Initial interest focused on the constitutively expressed bradykinin B₂ receptor, which is selectively activated with high affinity by bradykinin (BK) and kallidin (KD). Recently, there has been considerable interest in the inducible B₁ receptor, which is selectively activated by desArg⁹-bradykinin (DABK) and desArg¹⁰-kallidin (DAKD), the N-terminal arginine-cleaved metabolites of BK and KD (Leeb-Lundberg et al., 2005). The B₁ receptor is not normally expressed in most

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1 Current affiliation: Rinat Laboratories, Pfizer, Inc., South San Francisco, California.
2 Current affiliation: Avigen, Inc., Alameda, California.
3 Current affiliation: Myriad Genetics, Salt Lake City, Utah.
4 Current affiliation: Amgen, Inc., Cambridge, Massachusetts.

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ABBREVIATIONS: BK, bradykinin; KD, kallidin; DABK, desArg⁹-bradykinin; DALBK, desArg⁹-Leu⁴-bradykinin; DAKD, desArg¹⁰-kallidin; DALKD, desArg¹⁰-Leu⁴-kallidin; CFA, complete Freund’s adjuvant; P-gp, P-glycoprotein; CNS, central nervous system; ELN441958, 7-chloro-2-[3-[(9-pyrindin-4-yl)-3,9-diazaspiro[5.5]undecanecarbonyl]phenyl]-2,3-dihydro-isoindol-1-one; MEM, minimum essential medium; FBS, fetal bovine serum; IL, interleukin; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FLIPR, fluorometric imaging plate reader; LC/MS/MS, liquid chromatography-tandem mass spectrometry; MDCK, Madin-Darby canine kidney; MOPS, 3-(N-morpholino)propanesulfonic acid; PK, pharmacokinetic; AUC, area under the concentration-time curve; DRG, dorsal root ganglion; desArg¹⁰-HOE140, desArg¹⁰-Arg⁹-Hyp³, Thr³, Tyr³, Oic⁸-BK; SSR240612, (2R)-2-[[3R-3-(1,3-benzodioxol-5-yl)-3-[[6-methoxy-2-naphthyl][sulfonyl][amino][propanoyl]-amino]-3-[(2R,6S)-2,6-dimethyl(piperidinyl)[methyl]phenyl]-N-isopropyl-N-methyl[propanamide hydrochloride]; LF22-0542, N-[[4-(4,5-dihydro-1H-imidazo[2,1-b]pyridinyl)[methyl]phenyl]methyl-2-[[6-methoxy-2,6-dimethyphenyl][sulfonyl][methylamino][ethoxy]-N-methylacetamide fumarate.
tissues, but it is dramatically up-regulated following inflammation and tissue injury, and it does not readily desensitize. The low sequence homology between \( B_1 \) and \( B_2 \) receptors (36%) is consistent with the high degree of selectivity displayed by many agonists and antagonists (Leeb-Lundberg et al., 2005).

\( B_2 \) receptors from different species fall into two pharmacological classes (Regoli et al., 2001). The rodent-like class includes rat, mouse, hamster, and dog \( B_2 \) receptors, whereas the primate-like class includes the human, rhesus, pig, and rabbit receptors. The rodent \( B_1 \) receptors are potently activated by both DABK and DAKD, whereas DAKD has considerably higher affinity for the primate receptors than does DABK. Antagonist pharmacology also differs between these two classes. For example, the prototypical \( B_1 \) receptor antagonist \( \text{desArg}^9\text{Leu}^8\text{bradykinin} \) (DALBK) is a neutral antagonist of primate receptors, but it is a partial agonist at rodent receptors (Meini et al., 1996; MacNeil et al., 1997). In addition, several classes of small molecule \( B_1 \)-receptor antagonists exhibit marked potency differences between primate and rodent receptors (Kuduk et al., 2004; Ransom et al., 2004).

It has been proposed that the \( B_2 \) receptor is involved in acute inflammation, whereas the \( B_1 \) receptor is the primary kinin receptor mediating chronic inflammation (Perkins et al., 1993). In peripheral tissues, activation of newly formed \( B_2 \) receptors promotes the generation of inflammatory mediators, including prostanoids, nitric oxide, and cytokines, resulting in plasma extravasation, leukocyte trafficking, edema, and pain (McLean et al., 2000; Eisenbarth et al., 2004; Lawson et al., 2005; Lub-Endberg et al., 2005). \( B_1 \) receptors are constitutively expressed on primary afferent pain fibers, and they are up-regulated following nerve injury (Levy and Zochodne, 2000). Activation of neuronal \( B_1 \) receptors may contribute to neuropeptide release and subsequent neurogenic inflammation in peripheral tissues (McLean et al., 2000) as well as central sensitization at the level of the spinal cord (Pesquero et al., 2000). \( B_1 \) agonists injected dermally produce pain in humans, which is exacerbated by prior ultraviolet irradiation of the injection area (Eisenbarth et al., 2004).

\( B_2 \) receptor knockout mice have a pain phenotype implicating a role for this receptor in inflammatory and neuropathic pain. These \( B_2 \) receptor-deficient mice exhibit reduced responding in several experimental pain models relative to wild-type mice, including acute tests (hot-plate and tail-flick), following formalin, capsaicin, or complete Freund's adjuvant (CFA) injection into the paw, and in the partial sciatic nerve ligation model of neuropathic pain (Pesquero et al., 2000; Ferreiro et al., 2001; Porreca et al., 2006).

Peptide antagonists including DABK and \( \text{desArg}^9\text{HOE140} \) provided early pharmacological evidence in rodents implicating the role of the \( B_1 \) receptor in pain. Peptide antagonists are effective in early and late-phase licking following formalin injection into the paw (Ferreira et al., 2002), CFA-induced thermal and mechanical hyperalgesia (Fox et al., 2003), and carrageenan-induced mechanical hyperalgesia (Poole et al., 1999). Peptide antagonists also reduce thermal and mechanical hyperalgesia in models of neuropathic pain, e.g., in the rat chronic constriction nerve injury model (Levy and Zochodne, 2000).

More recently, small molecule \( B_1 \) receptor antagonists have shown efficacy in rodent pain models. SSR240612 reduced late-phase licking in the mouse formalin model, inhibited thermal hyperalgesia induced by ultraviolet irradiation in rats and also reduced thermal hyperalgesia in the rat chronic constriction nerve injury nerve injury model (Gougat et al., 2004). LF22-0542 reduced responding in the late phase of the formalin model and in the acetic acid-induced writhing model in mice, and it also reduced formalin-induced flinching, carrageenan-induced mechanical hyperalgesia, and CFA-induced thermal hyperalgesia in rats (Porreca et al., 2006). LF22-0542 reduced thermal, but not mechanical, hyperalgesia in the rat spinal nerve ligation model (Porreca et al., 2006).

In the present study, we describe the in vitro pharmacological profile, metabolic stability, P-glycoprotein (P-gp) liability, CNS penetration, in vivo pharmacokinetics, and primate analgesic efficacy of the novel \( B_1 \) receptor antagonist ELN441958 (Fig. 1).

**Materials and Methods**

**Synthesis of ELN441958.** Addition of tert-butyl 3,9-diazaspiro[5,5]undecane-3-carboxylate to 4-chloropyridine hydrochloride followed by deprotection in neat trifluoroacetic acid afforded 3-(4-pyridyl)-3,9-diazaspiro[5,5]undecane trifluoroacetate. Addition of methyl 3-aminobenzoate to methyl 2-(bromomethyl)-6-chlorobenzoate followed by ester hydrolysis gives 3-(7-chloro-1-oxo-1,3-dihydroisoindol-2-yl)benzoic acid, which is then coupled with 3-(4-pyridyl)-3,9-diazaspiro[5,5]undecane trifluoroacetate to afford 7-chloro-2-[3-(9-pyridin-4-yl)-3,9-diazaspiro[5,5]undecanecarbonyl phenyl]-2,3-dihydro-isoindol-1-one (ELN441958).

**Recombinant Cell Lines.** The complete open reading frame encoding the bradykinin \( B_1 \) receptor (BKBR1) is contained within a single exon in both the mouse and rat genomes. Accordingly, the cDNAs for each BKBR1 were amplified from mouse and rat genomic DNA (Clontech, Mountain View, CA) using primers specific for the 5' and 3' ends of the respective coding regions (GenBank, mouse: U47281; rat: U66107). Polymerase chain reaction amplification was achieved under standard conditions, using a combination of Taq and \( Pfu \) DNA polymerases for improved fidelity. Amplification products were subcloned into the TOPO TA cloning vector pCR 2.1 TOPO (Invitrogen, Carlsbad, CA) and sequenced. The correct BKBR1 cDNA was subcloned out of the TOPO TA vector and introduced into a PEAK 8 plasmid (Edge Biosystems, Gaithersburg, MD) that was previously modified by the addition of novel sites in the polylinker region by an overlapping oligonucleotide strategy. This plasmid is an episomal vector that drives transgene expression with the e\( \text{CMV} \) promoter, and stable clones can be selected with puromycin. The mouse and rat BKBR1 PEAK plasmids were stably transfected into PEAK-R and PEAK-S cells (Edge Biosystems), modified human embryonic kidney 293 cells designed specifically to preserve episomal vector expression. Stable populations of cells were maintained with puromycin. Stable transfectants of either PEAK-R or PEAK-S cells were selected based on their responsiveness in the cell-based assay (see below).

**Cell Culture.** IMR-90 human lung fibroblast cells (CCL-186; American Type Culture Collection, Manassas, VA) and DBS-FRhL-2 rhesus lung fibroblast cells (CL-160; American Type Culture Collection) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) as recommended.

![Structure of ELN441958](https://example.com/structure.png)
by American Type Culture Collection. Peak-S or -R cells expressing recombinant rat or mouse B1 receptors, respectively, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 400 μg/ml hygromycin B, and 1.5 μg/ml puromycin.

**Radioligand Binding.** Confluent IMR-90 cells were treated with 0.35 ng/ml interleukin (IL)-1β in 10% FBS/MEM for 4 h to up-regulate B1 receptors. Cells were then rinsed with phosphate-buffered saline, collected by scraping in ice-cold TES buffer (25 mM Na2TES, 1 mM phenantrone, 2 μM captopril, and 140 μg/ml bacitracin, pH 7.4), and centrifuged at 9200g for 5 min. Pellets were homogenized in TES buffer on ice using a polytron-type homogenizer (VirtiS, Gardiner, NY). The membranes were collected and washed by centrifugation twice at 50,000g for 20 min at 4°C. Pellets were resuspended in buffer at 0.5 mg/ml protein and stored at −80°C.

Protein concentration was measured using the biocinchonic acid method in the presence of 2% SDS (Pierce Chemical, Rockford, IL). Displacement binding experiments were carried out in 96-deep well plates in binding buffer [20 μM HEPS in Hanks’ balanced salt solution (HBSS) without bicarbonate or phenol red, 0.1% BSA, 1 mM phenantrone, 2 μM captopril, and 140 μg/ml bacitracin, pH 7.4] at room temperature. Approximately 40 μg of membranes/well were incubated with −0.5 nM [3H]DAKD (PerkinElmer Life and Analytical Sciences, Wellesley, MA) in the absence or presence of different concentrations of test compounds in a final volume of 300 μl. The actual [3H]DAKD concentration used in each experiment was determined by sampling the working solution of [3H]DAKD in binding buffer and counting at 37°C. The extent of binding was determined in duplicate wells (final 1% DMSO). Nonspecific binding was determined in the presence of 1 μM DAKD. After a 1-h incubation, binding was terminated by rapid filtration through GF/B filter plates (Whatman, Florkan Park, NJ) precoated in 0.2% polyethyleneimine using a Packard Filtermate (PerkinElmer Life and Analytical Sciences). Filters were rinsed four times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4), and bound radioactivity was counted after a 10-min incubation with 0.35 ng/ml human IL-1β (PeproTech, Rocky Hill, NJ) at 37°C and 5% CO2, and they were plated into 96-well plates (Costar 3904; Corning Life Sciences, Acton, MA) at approximately 13,000 cells/well. The following day, cells were treated with 0.35 ng/ml human IL-1β in 10% FBS/MEM for 2 h to up-regulate B1 receptors. Induced cells were loaded with fluorescent calcium indicator by incubation with 2.3 μM Fluo-4/acetoxyethyl ester (Invitrogen) at 37°C for 1.5 h in the presence of an ion transport inhibitor (2.5 mM probenzidine and 0.1% BSA in 20 mM HEPES/HBSS without bicarbonate or phenol red, pH 7.5), and cell plates were kept in the dark until used. Test compounds were assayed at seven concentrations in triplicate. Addition of test compounds to the cell plate and incubation for 5 min at 37°C, followed by the addition of 2 to 8 nM final (3 × EC50) B1 agonist DAKD (Bachem California, Torrance, CA), were carried out in the fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) while continuously monitoring calcium-dependent fluorescence. B1 responses were measured in IMR-90 cells in an identical manner except that the IL-1β treatment step was omitted and BK (0.7 nM final; 3 × EC50) replaced DAKD as agonist.

Cells expressing recombinant rat or mouse B1 receptors were detached using PBS without calcium or magnesium and loaded with 3.6 μM Fluo-4-acetoxyethyl ester for 75 min at 37°C in 1% FBS-containing HEPES/HBSS without bicarbonate or phenol red, pH 7.5. Loaded cells were washed by centrifugation, resuspended in assay buffer (0.1% BSA in HEPES/HBSS solution), and plated into 96-well black/white clear plates at 100,000 cells/well. Plates were centrifuged (200g; 10 min) to move cells to the bottom of wells, and then they were assayed in the FLIPR as described above, with DABK as agonist at 3 × EC50 concentration (4–10 nM). Peak height of agonist-induced fluorescence as a function of agonist concentration was fitted to the sigmoidal function (Prism; GraphPad Software Inc.) to determine IC50 values. Ki values were calculated according to the Cheng-Prossoff equation using the EC50 of [3H]DAKD (0.31 nM) determined in saturation binding experiments.

**Calcium Mobilization (FLIPR).** Human (IMR-90) and rhhesus (DBS-FRhL-2) lung fibroblast cells expressing native B1 receptors were harvested by trypsinization and seeded into black wall/clear bottom 96-well plates (Costar 3904; Corning Life Sciences, Wellesley, MA) while continuously monitoring calcium-dependent fluorescence. B2 responses were measured in IMR-90 cells containing HEPES/HBSS without bicarbonate or phenol red, pH 7.4, at room temperature, and centrifuged at 14,000g for 60 min. The concentration of ELN441958 in the supernatant was determined using high-performance liquid chromatography-UV (aqueous acetone containing 0.1% formic acid mobile phase) by comparing its peak area to a standard curve.

**Metabolic Stability.** ELN441958 (1 μM) was incubated at 37°C with rat or human liver microsomes (0.5 mg/ml; BD Biosciences, Bedford, MA) in the presence of 1 mM NADPH for 60 min. Aliquots were then incubated with 0.5, 1, 2, 3, 10, 20, 30, 45, and 60 min, precipitated with organic solvent containing internal standard, and analyzed by LC/MS/MS using a C18 column (2.1 × 50 mm, 5 μm; Sepax GPC18; Sepax Technologies, Newark, DE; flow 900 μl/min) with aqueous acetone containing 0.1% formic acid as mobile phase. The amount of parent compound remaining at each time point was estimated by the chromatographic peak area ratios and then plotted using a log-linear fit to determine the first-order rate constant. Midazolam, a high-clearance positive control, was incubated in parallel in each experiment.

**Permeability.** ELN441958 was evaluated for its permeability across a monolayer of Madin-Darby canine kidney (MDCK) II cells (American Type Culture Collection). Cells were grown for 4 days in Dulbecco’s modified Eagle’s medium with GlutaMAX containing 10% heat-inactivated FBS and 40 units/ml penicillin/streptomycin at 37°C and 5% CO2, and they were incubated into 12-mm-diameter 12-well Transwell plates (Corning Life Sciences). ELN441958 (5 μM) was added in HEPES/HBSS with 0.1% glucose, pH 7.4, and plates were incubated for 2 h at 37°C with shaking. Donor and receiver wells were then sampled and assayed by LC/MS/MS to determine compound levels. From these data, the forward flux (apical to basolateral) was calculated. The extent of transport by P-gp was determined in the same manner, except that MDCK cells expressing human multidrug resistance (MDR)-1 (kind gift from Dr. Piet Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) were used, and the forward flux was calculated in the absence or presence of the P-gp inhibitor cyclosporine A at 10 μM (Sigma-Aldrich, St. Louis, MO). The known P-gp substrate indinavir was used as a positive control. Integrity of the monolayer was determined using 100 μM Lucifer yellow (Sigma-Aldrich) with <2% leakage observed in all experiments.

**ATPase.** Membranes prepared from Sf9 insect cells expressing human MDR1 using the baculovirus expression system (Autographa californica) were purchased from BD Biosciences. The ATPase reaction was carried out in a 35-μl reaction volume in a 384-well microtiter plate containing 1% DMSO, 0.1 to 100 μM ELN441958, and 0.67 mg/ml Sf9 membranes in a reaction buffer consisting of 50 mM KCl, 50 mM MOPS-Tris, pH 7.0, with 5 mM sodium azide, 2 mM dithiothreitol, 0.1 mM EDTA, and 1 mM ouabain. The reaction was started with the addition of MgATP (5 mM final), incubated for 50 min at 37°C, and then quenched with SDS (2:2:2 final). The colorimetric reaction was developed for 40 min at 37°C following addition of the detection reagent consisting of 60 mM ammonium molybdate and 15 mM zinc acetate, and then the absorbance was determined at
800 nm using a Safire® microplate reader (Tecan, Durham, NC). Indinavir was used as a positive control.

**ELN441958 Formulation.** To achieve maximal exposure for pharmacokinetic and efficacy studies, ELN441958 was formulated as a clear solution. For rodent studies, ELN441958 was dissolved in 2% Tween 80 in saline and adjusted to pH 4.5 to 7 with HCl. For rhesus PK studies, ELN441958 was formulated in 10% polyethylene glycol 660 hydroxyethylate (Solutol; BASF, Ludwigshafen, Germany) in either saline (i.v.) or water (p.o.), adjusted to pH 4.5 to 7 with HCl. For s.c. administration in rhesus monkeys in both PK and efficacy studies, it was formulated as a clear solution in 20% Captisol (sulfobutylerthyl-β-cyclodextrin; CyDex, Lenexa, KS) in water adjusted to pH 4.5 to 7 with formic acid.

**Pharmacokinetic Analysis.** Aliquots of plasma (100 µl) from rhesus monkeys or Sprague-Dawley rats were added to extraction plates containing an internal standard in 400 µl of methanol/acetonitrile (50:50) and mixed. After centrifugation, the supernatant was transferred to a 96-deep well plate and evaporated to dryness. The residue was reconstituted in 20% methanol/0.1% formic acid mobile phase, and it was analyzed by LC/MS/MS using a C18 column and an API-3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Plasma concentrations were determined from a calibration curve prepared by extracting known amounts of ELN441958 from control plasma. The sensitivity limit was approximately 0.25 ng/ml or ng/g ELN441958 per sample.

**CNS Penetration.** To assess the contribution of P-gp to CNS penetration, the brain and plasma levels of ELN441958 in female FVB wild-type mice were compared with MDR1 a/b (−/−) knockout mice (Tacornic Farms, Germantown, NY) at 5 min following a single 2.5 mg/kg i.v. bolus injection. To assess the time course of CNS penetration, brain, spinal cord, and plasma levels were measured in male FVB wild-type mice at 0.5, 1, 2, and 4 h following s.c. administration of 10 mg/kg ELN441958. LC/MS/MS quantitation and tissue sample preparation were as described above, except that ELN441958 levels in brain and spinal cord were quantitated following homogenization of the preweighed tissue in ethyl acetate, and calibration curves were prepared by extracting known amounts of ELN441958 from ethyl acetate homogenates of control tissue. Mouse tissues were perfused, and the reported brain concentration was adjusted for the concentration of ELN441958 in plasma. The total area under the concentration-time curve from time 0 to 4 h (AUC0-4) was estimated from plasma, brain, and spinal cord concentrations using standard methods. These values were then used to calculate brain/plasma and spinal cord/plasma ratios.

**Carrageenan-Induced Hyperalgesia.** Adult male and female rhesus monkeys (Macaca mulatta) (weight range 9–11 kg; n = 5) were trained in the tail-withdrawal procedure described by Ko and Lee (2002). A 20-s maximal tail-withdrawal latency (baseline cut-off) was shown in Fig. 2. The potencies for stimulation of calcium mobilization by the appropriate agonist for B1 receptors from the different species examined, i.e., DAKD at the human and rhesus monkey B1 receptors and DABK at rat and mouse B1 receptors, are shown in Table 2. In IMR-90 cells expressing the native human B1 receptor, ELN441958 produced a concentration-dependent antagonism of the DAKD-induced calcium mobilization with a KD of 0.12 ± 0.02 nM (Figs. 4A and 5A; Table 3). ELN441958 itself does not produce an agonist response at concentrations completely blocking the agonist-induced response at all four species of receptor tested, indicating that it is a neutral antagonist in this assay (data for the rat receptor is shown in Fig. 4B). In contrast, the peptide antagonist DALKD exhibits clear agonism at rodent B1 receptors (Fig. 4B) as reported previously (Meini et al., 1996; MacNeil et al., 1997). A very small agonist response was generally observed following application of desArg1-HOE140 at high concentrations. The rank order potency for ELN441958 antagonism of B1 receptors from different species was human ∼ rhesus > rat > mouse; it was 120-fold less potent at the mouse relative to human receptor (Fig. 5; Table 3). In contrast, the peptide antagonists showed much less species difference in antagonist potency. The rank order potency for DALKD antagonism

**TABLE 1**

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<tr>
<th>Antagonist</th>
<th>Ki (nM)</th>
<th>Hill Slope</th>
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<tr>
<td>ELN441958</td>
<td>0.26 ± 0.02</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>DALKD</td>
<td>0.49 ± 0.06</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>desArg1-HOE140</td>
<td>15 ± 3</td>
<td>0.88 ± 0.07</td>
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whereas desArg1-HOE140 had more than 50-fold lower affinity. Saturation binding studies were performed to determine the type of inhibition (Fig. 3). KD and Bmax values (mean ± S.E.M.; n = 3) were 0.31 ± 0.03 nM and 1.8 ± 0.5 pmol/mg, respectively, in the control condition. In the presence of 0.5 nM ELN441958, KD and Bmax values were 1.3 ± 0.1 nM and 1.7 ± 0.4 pmol/mg, respectively, consistent with competitive inhibition.

**Results**

**Potency and Selectivity of ELN441958 at Bradykinin B1 Receptors.** ELN441958 inhibited the binding of the agonist radioligand [3H]DAKD to the human B1 receptor present in IMR-90 lung fibroblast cell membranes with high affinity (Ki = 0.26 ± 0.02 nM) (Fig. 2; Table 1). The peptide antagonist DALKD had a similar subnanomolar affinity, whereas desArg1-HOE140 had much less species difference in antagonist potency.
of B₁ receptors was rhesus ~ human > mouse ~ rat, with a 7-fold difference between rhesus and rat receptors. The less potent antagonist desArg¹⁰HOE140 showed very little species difference, a range of only 4-fold across the four species.

ELN441958 is highly selective for B₁ over B₂ receptors. In IMR-90 cells, ELN441958 failed to inhibit the calcium mobilization induced by the selective B₂-receptor agonist BK at concentrations up to 10 μM (data not shown). Likewise, desArg¹⁰HOE140 and DALKD were inactive at inhibit-
ing BK-induced responses up to 5 and 30 μM, respectively. In contrast, the selective B₂ receptor antagonist HOE140 potently inhibited the BK-induced response (IC₅₀ = 2.3 ± 0.4 nM). Regarding other nontarget receptors, 10 M ELN441958 produced ≥50% inhibition at 20 of more than 100 enzyme and receptor assays tested in a broad panel (data not shown). Of these, IC₅₀ values were determined for five receptors in single experiments. The most potent off-target activity identified was at the human μ-opioid receptor (binding Kᵢ = 0.13 μM), suggesting >500-fold selectivity for B₁ over μ-opioid receptors. Lower potency inhibition was measured at the human muscarinic M₁, δ-opioid, and κ-opioid receptors (binding Kᵢ values of 0.37, 0.69, and 1.5 μM). Because in vivo analgesia studies could be complicated by agonism at opioid receptors, ELN441958 was tested in guanosine 5′-O-(3-[35S]thio)triphosphate binding assays and found to be an agonist at the δ-opioid receptor (EC₅₀ = 0.76 μM) and also exhibited weak agonism at the μ-opioid receptor (<50% stimulation at 10 μM) (data not shown).

Metabolic Stability, Permeability, P-Glycoprotein Liability, and CNS Penetration. ELN441958 was sufficiently soluble (69 ± 16 μM) in aqueous buffer at pH 7.4 for further characterization of metabolic stability and permeability. ELN441958 demonstrated good metabolic stability with low in vitro intrinsic clearance in rat, rhesus, and human microsomes (Table 4). In contrast, the metabolically labile positive control midazolam was cleared much more rapidly. ELN441958 had moderate permeability in MDCK II cells (Table 5). However, the permeability of ELN441958 in MDCK cells expressing the recombinant human MDR1 gene coding for P-gp (MDR1-MDCK cells) was substantially lower. Cyclosporine A, an inhibitor of P-gp, increased the permeability in these cells 13-fold. The known P-gp substrate indinavir exhibited similar behavior in these assays, but it had substantially lower intrinsic permeability than ELN441958. In addition, ELN441958 stimulated P-gp ATPase activity 2-fold in membranes expressing the recombinant human MDR1 gene. Taken together, these data indicate that ELN441958 is a substrate for P-gp.

P-gp is one of the components of the blood-brain barrier,

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rat</th>
<th>Rhesus</th>
<th>Human</th>
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<tbody>
<tr>
<td>ELN441958</td>
<td>50 ± 2</td>
<td>43 ± 4</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>t₁/₂ (min)</td>
<td>28 ± 1</td>
<td>33 ± 3</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Intrinsic clearance (μL/mg/min)</td>
<td>1.6 ± 0.4</td>
<td>3.3 ± 1.3</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>Midazolam</td>
<td>1200 ± 400</td>
<td>650 ± 230</td>
<td>430 ± 100</td>
</tr>
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TABLE 4
ELN441958 has good in vitro metabolic stability across species.
Metabolic clearance determined by decay of parent compound incubated with microsomes as measured by LC/MS/MS. Data are means ± S.E.M. (n = 4).

Fig. 5. ELN441958 exhibits species differences in potency for antagonism of selective B₁ agonist-induced calcium mobilization. Antagonism of DAKD (human and rhesus) or DABK (rat and mouse)-induced calcium increase in cells expressing either native (human and rhesus) or recombinant (rat and mouse) B₁ receptors indicated by Fluo-4 fluorescence using FLIPR. Means ± S.E.M. of at least three experiments. Calculated Kᵢ values are shown in Table 3.
ELN441958 is a substrate for P-glycoprotein

See Materials and Methods for description of the assays. Values are means ± S.E.M. of at least three independent in vitro experiments or single in vivo studies (n = 3–4).

<table>
<thead>
<tr>
<th>Assay or Model</th>
<th>ELN441958</th>
<th>Indinavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp ATPase (-fold stimulation over basal)</td>
<td>2.1 ± 0.2</td>
<td>7.1 ± 0.9</td>
</tr>
<tr>
<td>P-gp ATPase EC₅₀ (nM)</td>
<td>N.A.*</td>
<td>15.1 ± 0.1</td>
</tr>
<tr>
<td>MDCK II permeability (nm/s)</td>
<td>54 ± 8</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>MDR1-MDCK permeability (nm/s)</td>
<td>7.8 ± 2.0</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>MDR1-MDCK permeability + cyclosporine A (nm/s)</td>
<td>110 ± 50</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>Cyclosporine A permeability ratio</td>
<td>13 ± 3</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Brain level in wild-type FVB mouse (µM)</td>
<td>&lt;0.002</td>
<td>0.042 ± 0.008</td>
</tr>
<tr>
<td>Brain level in MDR1 a/b (−/−) mouse (µM)</td>
<td>1.5 ± 0.3</td>
<td>0.40 ± 0.03</td>
</tr>
</tbody>
</table>

* Stimulation produced by ELN441958 was too low to calculate an EC₅₀ value.

** Percentage of recoveries for ELN441958 and indinavir were 44 ± 5 and 82 ± 3, respectively.

** Percent recoveries for ELN441958 and indinavir were 41 ± 4 and 59 ± 16, respectively.

** Plasma levels for ELN441958 and indinavir were 7.4 and 6.7%, respectively, of plasma exposure (Table 6).

** Plasma levels for ELN441958 and indinavir were 7.4 ± 0.5 and 3.2 ± 0.1 µM, respectively.

** Plasma levels for ELN441958 and indinavir were 7.4 ± 0.5 and 3.2 ± 0.1 µM, respectively.

and it is known to limit CNS penetration of certain compounds (Demeule et al., 2002). Because in vitro assays predicted that the CNS exposure of ELN441958 may be limited by P-gp, an in vivo study comparing brain levels in wild-type mice to MDR1 knockout mice was done. Five minutes after an i.v. dose of 2.5 mg/kg, ELN441958 achieved low micromolar brain levels in MDR1 knockout mice, but it was below the limit of quantitation in wild-type mice (Table 5), consistent with P-gp-mediated extrusion from the CNS. To further characterize the CNS penetration by this compound, a brain and spinal cord time-course study was performed in wild-type mice. As shown in Fig. 6, the levels of ELN441958 were considerably lower in brain and spinal cord relative to plasma at all of the time points measured. Based on the AUC ratios, ELN441958 exposure in the spinal cord and brain was 1.4 and 6.7%, respectively, of plasma exposure (Table 6).

**Pharmacokinetics.** ELN441958 exhibits a favorable pharmacokinetic profile in the rat (Fig. 7; Table 7). After an i.v. dose of 2.5 mg/kg, ELN441958 had a moderate volume of distribution (2.7 l/kg, approximately four times total body water) and a moderate clearance (0.85 l/h/kg, approximately 24% of hepatic blood flow). The terminal plasma half-life of this compound in rats was 1.7 h. When dosed orally, concentrations of ELN441958 increased to a maximum of 1.2 µg/ml at 2 h after dosing. The oral availability of ELN441958 in rats was 57%.

ELN441958 had an excellent pharmacokinetic profile in rhesus monkeys (Fig. 8; Table 7). When dosed i.v. at 1 mg/kg, ELN441958 had a moderate volume of distribution (2.7 l/kg) and a moderate clearance (0.49 l/h/kg, approximately 32% of hepatic blood flow). The terminal plasma half-life of this compound was 3.9 h in rhesus monkeys. After oral dosing, concentrations of ELN441958 increased to a maximum of 3.6 µg/ml at 3.3 h after dosing. The calculated oral bioavailability was greater than 100%. The pharmacokinetics of ELN441958 was also examined in rhesus monkeys by the s.c. route of administration to support efficacy studies. At an s.c. dose of 10 mg/kg, ELN441958 achieved peak plasma levels of 5.6 µg/ml at 2.2 h postdose. The calculated s.c. bioavailability was also greater than 100%.

**Carrageenan-Induced Hyperalgesia.** Because ELN441958 has lower potency at rodent than primate B₃ receptors, it was evaluated in a primate pain model. ELN441958 dose-dependently reduced the thermal hyperalgesia elicited by carrageenan injected into the tail (Fig. 9). In this tail-withdrawal procedure, carrageenan injection (2 mg/tail) produced a thermal hyperalgesia manifested by a reduced tail-withdrawal latency from 20 s (baseline) to 1 to 2 s in 46°C water. A 30-min pretreatment with 10 mg/kg ELN441958 s.c. completely blocked this hyperalgesia after 1 to 1.5 h postcarrageenan injection, and this antihyperalgesic effect was maintained for at least 3.5 h, consistent with sustained pla-
The benzamide ELN441958 is an optimized member of a new class of nonpeptide bradykinin B1 receptor antagonists.

Table 7. Pharmacokinetic parameters for ELN441958 in the rat and rhesus monkey

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat</th>
<th>Rhesus</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v. dose (mg/kg)</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>i.v. t1/2 (h)</td>
<td>2.0 ± 0.8</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>i.v. AUC0-t (µg · h/ml)</td>
<td>2.6 ± 0.3</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>i.v. Vz (l/kg)</td>
<td>2.7 ± 0.7</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>i.v. clearance (l/h/kg)</td>
<td>0.96 ± 0.12</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>Oral dose (mg/kg)</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Oral Cmax (µg/ml)</td>
<td>1.3 ± 0.5</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Oral T1/2 (h)</td>
<td>1.7 ± 0.6</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>Oral AUC0-t (µg · h/ml)</td>
<td>6.0 ± 1.4</td>
<td>14 ± 0</td>
</tr>
<tr>
<td>Oral bioavailability (%)</td>
<td>57</td>
<td>~100*</td>
</tr>
</tbody>
</table>

The oral and s.c. bioavailability of ELN441958 exceeded the maximum possible (100%), and they were calculated to be 128 ± 17 and 137 ± 40%, respectively.

Fig. 8. ELN441958 exhibits a favorable pharmacokinetic profile in the rhesus monkey. Male rhesus monkeys were dosed i.v. at 1 mg/kg in 10% polyethylene glycol 660 hydroxystearate/HCl/saline or orally at 5 mg/kg in 10% polyethylene glycol 660 hydroxystearate/HCl/water or s.c. at 10 mg/kg in 20% Captisol/water (n = 3). See Materials and Methods for analytical details. Facial flushing was noted in all animals 0 to 3 min following i.v. bolus injection, and in one of three animals 16 min after the s.c. injection.

Fig. 9. ELN441958 dose-dependently reduced carrageenan-induced hyperalgesia in rhesus monkeys. ELN441958 or vehicle was injected s.c. 30 min before local injection of carrageenan into the tail. Tail-withdrawal latencies in 46°C water were determined at 30-min intervals following carrageenan injection. Vehicle control data (open squares) partially superimposes on the 1 mg/kg dose (closed diamonds). Each symbol represents the mean ± S.E.M. for three subjects in a single dosing session.

Discussion

The benzamide ELN441958 is an optimized member of a new class of nonpeptide bradykinin B1 receptor antagonists.
The benzamide core was predicted to confer activity based on molecular modeling studies involving the crystal structure of a series of potent B₁ receptor antagonists, the 3,4-dihydroquinazoline (Grant et al., 2006). This modeling suggested that the quinazoline core and its substituents could be effectively replaced by a substituted benzamide, and initial compounds synthesized proved to be low micromolar B₁ antagonists. Initial studies involving the crystal structure of human bradykinin B₁ receptor. It has subnanomolar affinity for displacement of the agonist ligand [³H]DAKD. In saturation binding studies, ELN441958 reduced the apparent affinity of [³H]DAKD more than 4-fold with little change in receptor density, consistent with reversible, competitive displacement of the agonist radioligand from the human B₁ receptor. Other types of small molecule antagonists whose mode of binding has been investigated have also been shown to compete with agonist, including the sulfonamide SSR240612 (Gougat et al., 2004), the quinoxaline compound A (Ransom et al., 2004), and the N-methylacetamide LF22-0542 (Porreca et al., 2006). ELN441958 is also a subnanomolar antagonist of agonist (DAKD)-induced calcium mobilization in IMR-90 cells expressing the native human B₁ receptor. No agonist-like activity was detected by ELN441958 alone in calcium mobilization studies, indicating that ELN441958 is a neutral antagonist of the B₁ receptor. In the same cells, ELN441958 does not inhibit the activation of the human bradykinin B₂ receptor at concentrations up to 10 μM, showing that it is highly selective for B₁ over B₂ receptors. ELN441958 also displays good selectivity for B₁ over other receptors examined in a broad screening panel. It is >500-fold selective for the B₁ receptor over the human µ-opioid receptor, the most potent off-target activity identified.

ELN441958 has marked species differences for B₁ receptor antagonist potency compared with the peptidic B₁ receptor antagonists DALKD and desArg¹⁰OE140. Agonist-induced calcium mobilization experiments indicate that ELN441958 is 120-fold less potent at the mouse B₁ receptor relative to the human receptor. Similar species differences have been noted previously for other series of small molecule B₁ receptor antagonists, including the quinoxalines and the diaminopyridines (Kuduk et al., 2004; Ransom et al., 2004). These findings are consistent with the relatively low sequence homology between B₁ receptors from human and mouse (72%; Hess et al., 1996) or rat (73%; Jones et al., 1999). Because there is high sequence homology between the mouse and rat receptor (90%; Jones et al., 1999), it is surprising that ELN441958 is 10-fold more potent at the rat than mouse B₁ receptor. Of residues known to be involved in the binding of peptides or small molecule ligands (Ha et al., 2005), only Ile97 in the human sequence shows divergence between the corresponding locations in the rat (Ile107) and mouse (Val1104) sequences. Site-directed mutagenesis studies would be required to determine whether this conserved substitution can account for the species differences observed.

MDCK-MDR1 permeability and ATPase assays indicate that ELN441958 is a substrate for human P-gp. Consistent with these in vitro results, ELN441958 had low CNS penetration in the mouse. Antagonism of spinal B₁ receptors may be important to achieve full analgesic efficacy of an orally delivered antagonist as suggested by the efficacy of intrathecally delivered B₁ receptor antagonists in pain models (Fox et al., 2003; Conley et al., 2005). Alternatively, it is possible that brain penetration may lead to increased central side effects, because there is a high level of constitutive B₁ receptor expression in monkey brains (Shughrue et al., 2003), and the B₁ receptor is expressed in human brain (Raidoo and Bhoola, 1997). Nevertheless, peripherally acting peptidic B₁ receptor antagonists given systemically are effective in pain models. For example, desArg¹⁰OE140 is effective against CFA-induced mechanical allodynia by either the intrathecal or s.c. route (Fox et al., 2003). An antagonist that is excluded from the spinal cord could still block B₁ receptors present on peripheral terminals of sensory fibers, and it may also gain access to receptors present on cell bodies in the dorsal root ganglion (DRG).

ELN441958 possesses a favorable pharmacokinetic profile as predicted by in vitro assays. ELN441958 is well absorbed after oral administration in rats and rhesus monkeys consistent with the moderate solubility and permeability in MDCK cells. In particular, ELN441958 is essentially completely absorbed and produces high plasma levels after oral adminis-

Fig. 10. The opioid-receptor antagonist naltrexone does not affect the antihyperalgesic effect of ELN441958 in the rhesus carrageenan model. Naltrexone (1 mg/kg s.c.) was injected just before ELN441958 (10 mg/kg s.c.). The vehicle control and ELN441958 alone data are the same as in Fig. 9. Each symbol represents the mean ± S.E.M. for three subjects in a single dosing session.

Fig. 11. Naproxen reduces carrageenan-induced hyperalgesia in rhesus monkeys. Naproxen (10 mg/kg s.c.) was injected 30 min before local injection of carrageenan into the tail. See Fig. 9 and Materials and Methods for details. Each symbol represents the mean ± S.E.M. for three individuals in a single dosing session.
tation in rhesus monkeys. The bioavailabilities calculated following oral and s.c. administration in monkeys were greater than 100%, possibly due to lack of dose proportionality by one or more route of administration as different dose levels used for each route. ELN441958 has a moderate clearance and volume of distribution in both species following i.v. administration, consistent with the high metabolic stability in rat, rhesus, and human microsomes. The high oral bioavailability and half-life of ~4 h in rhesus monkeys coupled with the good metabolic stability in human microsomes predict a favorable pharmacokinetic profile for ELN441958 in humans.

Several approaches have been taken to demonstrate efficacy for B₁ receptor antagonists with low potency at rodent B₁ receptors, precluding their evaluation in standard rodent pain models. For example, Su et al. (2003) exploited the pharmacological similarity between primate and rabbit B₁ receptors by demonstrating the potent efficacy of a quinoxaline B₁ receptor antagonist in a CFA-induced hyperalgesia in rabbits. In an elegant study by Fox et al. (2005), the highly human-selective B₁ receptor antagonist NVP-SAA164 reversed CFA-induced hyperalgesia in transgenic mice expressing only the human B₁ receptor. An alternative approach is to evaluate compounds in primate pain models.

Use of primate pain and inflammation models to evaluate potential therapeutic agents is advantageous for several reasons. In addition to species differences in receptor potency, a clear advantage of the use of primate models is the more direct applicability to humans. There are often major differences in the pharmacology of neurotransmitter systems involved in pain transmission between humans and rodents, e.g., cholecystokinin (Hökfelt et al., 2001) and substance P (Hill, 2000). In addition, scaling from monkey pharmacokinetic data is generally the most accurate method to predict human clearance (Evans et al., 2006). In the bradykinin system, human and monkey B₁ receptors are functionally indistinguishable, whereas rodent receptors exhibit a different agonist and antagonist pharmacology (Regoli et al., 2001). Demonstration of efficacy in primates using experimental analgesic agents with novel mechanisms of action not validated clinically increases the likelihood of analgesic efficacy in humans.

The rhesus monkey tail-withdrawal procedure is a well-characterized method for assessing antinociception in primates. Initially established as an acute pain model, the procedure involves measuring the latency to withdrawal of the tail immersed in water at an acutely noxious temperature (e.g., 50°C) (Ko et al., 2004). Trained monkeys respond to standard analgesic agents such as opioids with an increased latency to tail withdrawal. To evaluate analgesics with a broader range of mechanisms, the procedure has been modified by pretreatment with agents that sensitize the tail to thermal stimuli. For example, topical application of capsaicin produces a gradual and relatively prolonged hyperalgesia, and it closely mimics the experimental use of capsaicin in humans (Butelman et al., 2003). Alternatively, carrageenan injection into the tail of rhesus monkeys produces a thermal hyperalgesia manifested as a reduced tail-withdrawal latency to a normally non-noxious water temperature (Ko and Lee, 2002). The latency to withdrawal in 46°C water is reduced to 1 to 2 s from a baseline of 20 s within 1 h after carrageenan injection and is maintained for at least 3.5 h.

ELN441958 dose-dependently reduces carrageenan-induced thermal hyperalgesia in rhesus monkeys, with a minimal effective dose of 3 mg/kg s.c. At this dose, ELN441958 does not affect the initial hyperalgesia produced by carrageenan, but it reduces hyperalgesia beginning 2 h after carrageenan injection, and it completely blocks the hyperalgesia by 3.5 h postinjection. The delayed antihyperalgesic effect observed at 3 mg/kg ELN441958 s.c. may be due to a progression of inflammatory mediators producing hyperalgesia following carrageenan injection. Specifically, generation of prostanooids may play a dominant role in carrageenan-induced hyperalgesia after about 2 h postcarrageenan injection, but not earlier. This idea is supported by a similar time-dependent reduction in hyperalgesia observed with the nonselective cyclooxygenase inhibitor naproxen at 10 mg/kg s.c., which probably reflects the time course for prostanooid production caused by carrageenan. The time-dependent reduction in carrageenan hyperalgesia observed at the 3 mg/kg dose of ELN441958 probably represents antagonism of B₁ receptor-mediated prostanooid production.

The early carrageenan-induced hyperalgesia may be unrelated to prostanooid production, and it is thus not sensitive to treatment with 10 mg/kg naproxen s.c. It has been shown that during the first hour following carrageenan injection into the rat paw, edema develops that is mediated by histamine, serotonin, and bradykinin release (Salvemini et al., 1996). Subsequently, neutrophil infiltration and production of NO, tumor necrosis factor-α, interferon-γ, cytokines (IL-1 and IL-2), and prostaglandins contribute significantly to the edema 1 to 6 h after carrageenan injection. In particular, NO production has been shown to dramatically increase within 1 h, whereas prostaglandin E₂ levels increase slowly, peaking at 3 h after carrageenan injection (Salvemini et al., 1996). Thus, it is likely that the high dose of 10 mg/kg ELN441958 s.c. reduces early carrageenan-induced hyperalgesia by mechanisms other than prostanooid production. Because B₁ agonists promote generation of NO (Sangsree et al., 2003), cytokine release (Tsukagoshi et al., 1999), and neutrophil infiltration (Ehrenfeld et al., 2006), B₁ antagonists may reduce the hyperalgesia elicited by these proinflammatory factors.

The potential contribution of receptor induction to the antihyperalgesic effects of ELN441958 in the carrageenan model must be considered. The peripheral non-neuronal effects of B₁ antagonists require receptor induction as receptor expression is normally very low or nondetectable. B₁ receptor induction in peripheral tissues can occur rapidly following tissue insult. For example, B₁ receptor mRNA is dramatically increased in the paw within 1 h following paw injection of IL-1β (Campos et al., 2002). Carrageenan paw injection increases B₁ mRNA paw expression at 3 h in rats (J. E. Hawkinson, unpublished observations). Taken together, these data suggest that carrageenan injection may rapidly (i.e., within about 1 h) induce B₁ receptors in peripheral tissues. In contrast to non-neuronal tissues, constitutive expression of the B₁ receptor in nociceptors has been demonstrated (Wotherspoon and Winter, 2000). Paw injection of CFA results in up-regulation of B₁ receptor in nociceptors has been demonstrated (Wotherspoon and Winter, 2000). Paw injection of CFA results in up-regulation of B₁ receptor in nociceptors has been demonstrated (Wotherspoon and Winter, 2000). Paw injection of CFA results in up-regulation of B₁ receptor in nociceptors has been demonstrated (Wotherspoon and Winter, 2000).
Rapid effects of 10 mg/kg ELN441958 observed within 1 h after carrageenan injection may require blockade of constitutive B₃ receptors expressed on pain afferents. Consistent with this idea, ELN441958 (3 and 10 mg/kg s.c.) produced a marked reduction in topically applied capsaicin-induced alldynia in rhesus monkeys within 5 min after removal of capsaicin. Antiallodynic activity of ELN441958 was observed in two of the four rhesus monkeys tested in a tail-withdrawal procedure using both 38 and 42°C stimulation (E. R. Butelman, unpublished observations). Because ELN441958 has poor penetration into the spinal cord, the antihyperalgesic activity at low doses is more likely mediated by B₁ receptors expressed on nociceptors at their peripheral terminals or at the cell bodies in the DRG, although high doses may be sufficient to penetrate the spinal cord and exert a central antihyperalgesic effect. Intrathecally administered B₁ receptor antagonists are effective in acute and inflammatory pain models (Fox et al., 2003; Conley et al., 2005).

Plasma levels achieved by ELN441958 at efficacious doses were substantially greater than its in vitro potency. This difference may be explained by a number of factors related to the compound and/or the receptor. For example, ELN441958 has restricted access to neuronal B₁ receptors expressed in spinal cord and access to receptors expressed on cell bodies in the DRG may also be limited. Accessibility to peripheral B₁ receptors may also be restricted if the compound is highly bound to plasma proteins. In addition, time-dependent up-regulation of B₁ receptor expression and the progression of inflammatory mediators with varying sensitivities to modulation by B₁ receptor antagonism following carrageenan injection may also contribute to the relatively high doses of ELN441958 required for efficacy.

Because ELN441958 has low affinity for μ- and δ-opioid receptors (i.e., it is >500- and >2000-fold selective for the human B₁ receptor over μ- and δ-opioid receptors, respectively), opioid receptors may play a minimal role in its antihyperalgesic effects. This notion is further supported by the finding that naltrexone 1 mg/kg s.c. was inactive in blocking the antihyperalgesic effects of ELN441958. Based on the binding affinity of naltrexone in monkey brain membranes and its in vivo antagonist potency in rhesus monkeys (Emmerson et al., 1994; Ko et al., 1998), this dose of naltrexone is sufficient to antagonize μ-, κ-, or δ-opioid receptor-mediated behavioral effects in monkeys. Although in this study we did not conduct a control experiment to show blockade of opioid receptor-mediated effects by this dose of naltrexone, several studies have shown that much smaller doses (i.e., 0.0032–0.1 mg/kg) antagonized a variety of opioid-mediated behavioral effects in rhesus monkeys (Ko et al., 1998, 2004; Bowen et al., 2005). For example, naltrexone 0.1 mg/kg produced a 10-fold rightward shift of the dose-response curve for μ-opioid agonist-induced antinociception (Ko et al., 1998). Taken together, it is unlikely that the opioid receptors significantly contribute to the antihyperalgesic effects of ELN441958 in monkeys.

In summary, ELN441958 is an optimized lead compound of a novel class of bradykinin B₁ receptor antagonists. ELN441958 is a potent, competitive, neutral antagonist of the human B₁ receptor, with lower potency at rodent B₁ receptors. It has a favorable pharmacokinetic profile in rats and rhesus monkeys as predicted by in vitro metabolism and permeability assays. ELN441958 is a P-gp substrate, consistent with low CNS exposure. ELN441958 reduced carrageenan-induced hyperalgesia in rhesus monkeys, probably acting primarily at peripheral B₁ receptors. Validation of the therapeutic utility of bradykinin B₁ antagonists in the treatment of human pain states will require evaluation in clinical trials.

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International Association for the Study of Pain (IASP) Press, Seattle, WA.


Address correspondence to: Dr. Jon E. Hawkinson, Lead Discovery and Optimization, Elan Pharmaceuticals, 800 Gateway Blvd., South San Francisco, CA 94080. E-mail: jon.hawkinson@earthlink.net