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Antinociceptive Effects of AS1892802, a Novel Rho Kinase Inhibitor, in Rat Models of Inflammatory and Non-inflammatory Arthritis

Eiji Yoshimi, Fumiyo Kumakura, Chie Hatori, Emi Hamachi, Akinori Iwashita, Noe Ishii, Takeshi Terasawa, Yasuaki Shimizu, Nobuaki Takeshita

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Address correspondence to: Eiji Yoshimi

Pain Research, Pharmacology Research Labs.

Astellas Pharma Inc.

21 Miyukigaoka, Tsukuba-shi, Ibaraki, 305-8585, Japan.

Tel: +81-29-829-6141

Fax: +81-29-852-5391

E-mail: eiji.yoshimi@jp.astellas.com

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Abbreviations: OA, osteoarthritis; AIA, adjuvant-induced arthritis; MIA, monoiodoacetate-induced arthritis; PGE2, prostaglandin E2; NSAID, non-steroidal anti-inflammatory drug

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Abstract

Rho kinase (ROCK) is involved in various physiological functions, including cell motility, vasoconstriction, and neurite extension. Although a functional role of ROCK in nociception in the central nervous tissue has been reported in neuropathy, the peripheral function of this protein in hyperalgesia is not known. In this study, antinociceptive effects of AS1892802, a novel and highly selective ROCK inhibitor, were investigated in two rat models of arthritis. Orally administered AS1892802 exhibited potent antinociceptive effect in both an adjuvant-induced arthritis (AIA) model (inflammatory arthritis model) and a monoiodoacetate-induced arthritis (MIA) model (non-inflammatory arthritis model), with an ED$_{50}$ of 0.15 mg/kg (MIA model). Fasudil, a ROCK inhibitor, and tramadol were also effective in both models; however, diclofenac was effective only in the AIA model. The onset of antinociceptive effect of AS1892802 was as fast as those of tramadol and diclofenac. AS1892802 did not induce gastric irritation or abnormal behavior. As AS1892802 rarely penetrates the central nervous tissue and is also effective by intra-articular administration, AS1892802 seemed to function peripherally. These results suggest that AS1892802 has an attractive analgesic profile for the treatment of severe osteoarthritis pain.
Introduction

Osteoarthritis (OA) is a cartilage degenerative disease and the most common type of arthritis, with high prevalence especially in elderly populations (Peat et al., 2001). Those patients suffer from pain, and OA pain leads to a remarkable deterioration in their quality of life (Briggs et al., 1999). Although analgesics, including acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), and opioids, are referenced as useful agents in the guideline for OA treatment, no drugs satisfy both efficacy and safety (Zhang et al., 2008). Total hip or knee joint arthroplasty is generally recommended in the case of inadequate pain relief in those joints.

In affected OA joints, various tissue disorders are detected, including cartilage breakdown, osteophyte formation, synovial hypertrophy, and bone sclerosis. These morphological changes are occasionally utilized as a diagnosis index as well as pain symptoms (Hunter and Felson, 2006). In spite of enormous research efforts, the origin of OA pain is still unclear. These structural changes are putative causes of pain; however, both accordance and discordance between the pain intensity and the severity in morphological changes have been reported (Creamer et al., 1999; Felson et al., 2001; Bedson and Croft, 2008; Hannan et al., 2000). Tissue inflammation is also likely to contribute to OA pain (Hill et al., 2001). However, the role of inflammation is supposedly limited, as efficacy of NSAIDs is partial in some patient populations (Bjordal et al., 2004) and severe synovial inflammation is detected in only 31%
of knee or hip joints extirpated from OA patients (Haywood et al., 2003). These facts suggest that OA patients have not only inflammatory pain but also non-inflammatory pain. Therefore, an agent that can control both types of pain would presumably be a preferable analgesic for the treatment of OA pain.

Rho kinase (ROCK), 160 kDa serine-threonine kinase, is the effector of Rho, a small GTP-bound protein (Ishizaki et al., 1996; Shimokawa and Rashid, 2007). Two isoforms, ROCK1 (ROKβ) and ROCK2 (ROKα), are known. The amino acid sequence homology between them is 65% in total, and 92% in their catalytic domain. Although the tissue distributions of ROCKs are regarded as ubiquitous, strong expression is known to occur in the heart, lungs, liver, stomach, spleen, kidneys, testes, and placenta for ROCK1, and in the brain, heart, lungs, muscles, and placenta for ROCK2 (Nakagawa et al., 1996). ROCKs control the cytoskeletal mobility and exert their physiological functions such as cellular shape change, contraction, or migration through the phosphorylation of downstream proteins such as LIM Kinase (LIMK) or Myosin Light Chain Kinase (MLCK) (Shimokawa and Rashid, 2007). Due to these multiple functions, a ROCK inhibitor has been applied for the treatment of various diseases. Fasudil, the sole clinically available ROCK inhibitor, is already in use to treat cerebral vasospasm, and its anti-angina effect has also been confirmed in humans (Vicari et al., 2006).

ROCK is also known to be involved in neuronal functions such as axonal extension or plasticity, and intrathecally injected ROCK inhibitors improved
alldynic condition in nerve injury models (Inoue et al., 2004; Ramer et al.,
2004; Tatsumi et al., 2005). Although an analgesic effect has been
demonstrated by intraperitoneal injection of the ROCK inhibitor Y-27632 in the
mouse hot plate test and acetic acid-induced writhing test (Büyükafşar et al.,
2006), the effect of ROCK inhibitors in an osteoarthritis model has not yet been
investigated.

In this study, the analgesic effects of novel ROCK inhibitor AS1892802 were
investigated in two rat models of chronic arthritis: an adjuvant-induced arthritis
(AIA) model (inflammatory arthritis model) and a monoiodoacetate-induced
arthritis (MIA) model (non-inflammatory arthritis model).
Methods

Animals

Six-week-old male Sprague-Dawley (SD) rats and eight-week-old female Lewis rats were obtained from Charles River Laboratories (Yokohama, Japan). Rats were housed under conditions that included a controlled light cycle (light/dark: 12 h each) and controlled temperature (23 ± 1 °C). Tap water and standard laboratory chow were available ad libitum. Rats were allowed to habituate themselves to the housing facilities for at least 3 days before agent treatments or behavioral testing. Experiments were conducted in accordance with the guidelines for Research and Ethical Issues of International Association for the Study of Pain (IASP) on using laboratory animals (Zimmermann, 1983). All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc.

Drugs

AS1892802 was synthesized by Astellas Pharma Inc. (Tsukuba, Japan). Fasudil, naloxone, indomethacin, and diclofenac sodium were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), morphine hydrochloride from Dainippon Sumitomo Pharma (Osaka, Japan), tramadol hydrochloride from Fluka AG (Buchs, Switzerland), and latrunculin A from Calbiochem-Novabiochem (Nottingham, UK). Fasudil, diclofenac, and tramadol were dissolved in distilled water, and naloxone was dissolved in saline.
AS1892802 was dissolved in distilled water containing equal mol-concentration of hydrochloride. Indomethacin was suspended in 0.5% methylcellulose. Test drugs were administered to rats orally at a volume of 5 ml/kg, or subcutaneously at a volume of 1 ml/kg.

**Behavioral Tests**

**MIA Model**

Chronic MIA rats were utilized as non-inflammatory arthritic pain model (Fernihough et al., 2004). Male SD rats were injected with 1 mg of MIA (Sigma, St. Louis, MO, USA; 50 μL in saline) into the right knee, and saline was injected in sham group rats (Bove et al., 2003). Analgesic tests were conducted on between Days 21 and 28 after MIA injection by Bove’s method of weight distribution using an automatic incapacitance tester (Linton Instrumentation, Norfolk, UK). Analgesic effects of test drugs were measured at 2 h after oral administration, except for in the time course experiment of AS1892802 (2, 6, 24 h). In the case of intra-articular administration, 3 μg of AS1892802 (in 50 μL of saline containing 10% polyethylene glycol 400) or vehicle was injected into the right (ipsilateral) or left (contralateral) knee 30 min before the analgesic measurements.

**Complete Freund’s AIA Model**

Chronic AIA rats were utilized as a typical inflammatory arthritic pain model
(Colpaert, 1987). Analgesic activity in AIA rats was measured using Magari’s modified method of Randall-Selitto (Magari et al., 2003; Randall and Selitto, 1957). Arthritis was induced in female Lewis rats by injection (50 μL) of a suspension of *Mycobacterium tuberculosis* H37Ra strain (0.5 mg, Difco Laboratories, Detroit, MI, USA) in liquid paraffin into the right hind foot pad. Pain threshold in the left hind paw was assessed using an algesimeter (Ugo Basile, Varese, Italy) between Days 18 and 21 after adjuvant inoculation. Test drugs were administered to rats orally (AS1892802, diclofenac, fasudil, tramadol, and morphine) or subcutaneously (naloxone) at the indicated time before the measurements. Rats in the control group were dosed with distilled water orally, or with saline subcutaneously. For topical injection, latrunculin A (500 ng in 50 μL of saline containing 0.5% dimethyl sulphoxide) or AS1892802 (3 μg in 50 μL of saline containing equal mol-concentration of hydrochloride) was intradermally injected into the left hind paw.

**Locomotor Activity**

Normal male SD rats were administered test drugs orally, and 1 h later the locomotor activity was measured in open field (33 × 44 × 32 cm) for 60 min with Supermex (Muromachi Kikai, Tokyo, Japan), an activity-monitoring system, by detecting the radiant body heat of rats (Masuo et al., 1997).

**Paw Volume Measurement**
Chronic AIA rats were given daily oral administration of AS1892802 (10 mg/kg) or indomethacin (1 mg/kg) for 6 days. Paw volume of the left hind paw was measured using a volume meter (Muromachi Kikai, Tokyo, Japan) before and after the repeated dosing (Magari et al., 2003).

**Prostaglandin E2 (PGE2) in the Inflamed Paw**

AIA was established in the same way as for the pain measurements described above. PGE2 in the paw was measured by the modified method, as described previously (Magari et al., 2003). Briefly, the left hind paw of each rat was amputated above the ankle joint, snap-frozen in liquid nitrogen, and stored at −80 °C until used. The paw tissues were added to 7 ml/g of lysis solution containing 80% methanol, 20% saline, and 10 μM indomethacin. The tissues were homogenized on ice using a Polytron homogenizer (PT10-35, Kinematica, Switzerland) and incubated on ice for 1 h. Supernatants collected by centrifugation at 5000 × g for 10 min were purified with C18 SEP-PAK cartridges (Waters, Milford, MA, USA) following the methods mentioned before (Powell, 1980). The eluents were freeze-dried and the dried pellets were dissolved in an assay buffer of PGE2 EIA kit (Cayman Chemical, Ann Arbor, MI). The concentration of PGE2 was determined by EIA following the manual of the EIA kit.
For BP measurement, rats were anesthetized with pentobarbital sodium (60 mg/kg ip), and a polyethylene cannula (PE-50) filled with heparin-saline solution was inserted via the left common carotid artery. The other end of the cannula was guided externally through the dorsal cervical region. After operation, the rats were housed individually in cages and used in experiments after a recovery period of at least 2 days. To measure the BP of each animal under conscious conditions, the rats were placed individually in cages, and the cannula was connected to a pressure transducer (TP-400T; Nihon Kohden, Tokyo, Japan) via a swivel (Instech Laboratories, Plymouth Meeting, PA, USA). AS1892802 or vehicle were orally administered, and BP was continuously recorded before and at 0.5, 1, 1.5, 2, 4, and 6 h after oral administration of AS1892802 or vehicle.

**Effect on Gastric Mucosa**

Male SD rats were orally administered test drugs once a day for a week. Five hours after the final dosing, animals were sacrificed and the stomachs removed and opened from the pyloric region along the greater curvature. The presence or absence of visible mucosal ulceration (≥ 1 mm) was noted, and the lengths of all ulcers were measured in millimeters, with values summed to provide a gastric damage score for each rat (Wallace et al., 2000).

**Recombinant Kinase Assay**

The inhibitory effects for human recombinant ROCK1 (hROCK1; Carna
Biosciences Inc., Kobe, Japan), human recombinant ROCK2 (hROCK2; CycLex Co., Nagano, Japan), and rat recombinant ROCK2 (rROCK2; Upstate Biotechnology, Lake Placid, NY, USA) were assayed by enzyme-linked immunosorbent assay. Briefly, myosin phosphatase targeting subunit 1 (MYPT1) (2.2 mg/ml, Upstate Biotechnology), substrate for ROCK, was diluted with a coating buffer (0.1 M NaHCO₃, pH 9.5) to 1:4000 for hROCK2 and rROCK2, and 1:8000 for hROCK1. Those diluents (50 μL) were added in 96-well plates and then the plates were incubated at 4 °C overnight. After washing twice with washing buffer (Tris Buffer pH 7.4, 0.1% Tween), the plates were blocked for 2 h with blocking buffer (washing buffer containing 1% bovine serum albumin). After washing twice, the assay buffer (5 mM MgCl₂, 20 mM MOPS, 25 mM beta-glycerophosphatase, 5 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate), ATP (10 μM for hROCK1 and rROCK2, 100 μM for hROCK2) and diluted kinases (10 ng/ml of hROCK1, 80 mU/ml of hROCK2, or 20 mU/ml of rROCK2) were added to wells to start the reaction (final volume 50 μL). After incubation at 37 °C for 60 min, the plates were washed and phosphorylated. Phosphorylated MYPT1 was detected using the first antibody (anti-phospho-MYPT1 [Thr696] antibody; Upstate Biotechnology) and the second antibody (HRP-Goat Anti-Rabbit IgG [H+L]; Amersham Biosciences Inc., Piscataway, NJ, USA). Horseradish peroxidase activity was detected using a TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD, USA). OD450 nm was measured on a SPECTRAMAX Microplate Reader (Molecular
Devices, Sunnyvale, CA, USA). IC$_{50}$ values and 95% confidence limits were calculated using GraphPad Prism (version 4, GraphPad Software Inc., San Diego, CA, USA) software.

**SH-SY5Y Cell Assay**

Promotive effects on neurite outgrowth *in vitro* were measured as described previously (Price et al., 2005). SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA) were plated onto 6-well plates at $6 \times 10^4$ cells/well and treated with 0.4 $\mu$M aphidicolin (in DMEM/15% fetal bovine serum/1% penicillin/streptomycin by volume) for five days. Cells were then treated with AS1892802 maintained in media containing 0.4 $\mu$M aphidicolin. For analysis of process length, cells (at least 20/well) were selected and photographed at 96 h. Neurite lengths were measured using WinRoof software (Mitani Corporation, Fukui, Japan) running on an IBM XT computer; only processes more than twice the cell body length and only one process per cell were measured.

**Statistical Analysis**

Data were analyzed and plotted on graphs using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA, USA). Results are expressed as mean ± S.E.M. Statistical analysis was performed using Student's t-test for comparison of two groups and one-way analysis of variance followed by Dunnett's multiple comparisons post-test for comparison of more than two
groups. In the time-course measurement study, analyses were conducted using two-way repeated-measures analysis of variance followed by Bonferroni’s post tests. The difference was considered statistically significant when $p < 0.05$. ED$_{50}$ values were calculated by the least squares method. (The indicated dose of test drugs was changed to log value and was applied to a simple linear regression).
Results

**AS1892802 is a potent and selective inhibitor of ROCK**

To find novel ROCK inhibitors, we subjected our in-house chemical libraries to large-scale high-throughput screening and obtained a lead compound (IC$_{50}$= 68 nM for hROCK1), which also potently inhibited human CYP isoforms (IC$_{50}$ values were <0.1, <0.1, >10, <0.1, and 0.25 μM for 3A4, 2D6, 1A2, 2C9, and 2C19, respectively). Systematic modification of the lead compound produced a novel, selective, and orally available compound, AS1892802 (Fig. 1) which potently inhibited hROCK1, hROCK2, and rROCK2 with IC$_{50}$ values (95% confidence interval) of 122 (93-160), 52 (36-76), and 57 (41-80) nM, respectively (Fig. 2A). Inhibition of CYP isoforms was remarkably diminished for AS1892802 (IC$_{50}$ values were 15, 17, >25, 13, and 19 μM for 3A4, 2D6, 1A2, 2C9, and 2C19, respectively). The IC$_{50}$ values of fasudil were 87 (61-124) nM (hROCK1) and 72 (45-116) nM (rROCK2) (data not shown). The ATP-concentration-dependent inhibitory activities on hROCK1 suggested that both compounds are ATP-competitive inhibitors (Fig. 2B). Among 167 kinases in the kinase panel assay (Carna Biosciences Inc., Kobe, Japan), 10 kinases were inhibited more than 50%, and only two kinases (cAMP-dependent protein kinase catalytic subunit [PKAC]-α and protein kinase X gene [PRKX]) were inhibited more than 80% at 10 μM of AS1892802 (Supplemental Table 1). The IC$_{50}$ values for those two kinases were larger than that for ROCK (PKAC-α: 200 nM; PRKX: 325 nM; data not shown). No remarkable affinity was detected for
63 proteins, including various ion channels, receptors, and enzymes at 10 μM (Caliper Life Sciences, Hopkinton, MA, USA; Supplemental Table 2).

**AS1892802 shows analgesic effect in chronic pain models**

The analgesic effects of AS1892802 were evaluated using models of both non-inflammatory and inflammatory chronic arthritic pain (rat MIA and AIA models, respectively).

With regard to established hyperalgesia in MIA rats, oral therapeutic administration of AS1892802 and a weak opioid (tramadol) induced an analgesic effect with ED$_{50}$ (95% confidence interval) values of 0.15 (0.10-0.23) mg/kg and 14 (7.2-27) mg/kg, respectively, whereas administration of NSAID (diclofenac, up to 30 mg/kg) did not (Fig. 3). Fasudil also exhibited an analgesic effect, but the potency (ED$_{50}$= 4.4 mg/kg, 95% confidence interval: 1.7-11) was approximately 30-fold weaker than that of AS1892802 (Fig. 3B). The effect of AS1892802 was clearly more potent than those of the other three drugs, and the weight imbalance disappeared completely at 1 mg/kg (Fig. 3A). When AS1892802 (1 mg/kg) was administered to MIA rats, the maximum plasma concentration (unchanged form) was 420 ng/ml at 0.8 h after administration, and the plasma concentrations at 2, 6, and 24 h were 285, 225, and 1.8 ng/ml, respectively. The time course of the analgesic effect of AS1892802 was well correlated with the plasma concentration level of the unchanged form (Fig. 3E).
For chronic inflammatory pain in a rat AIA model, diclofenac demonstrated an analgesic effect on the mechanical stimulus in a dose-dependent manner, with the minimum effective dose of 1 mg/kg (Fig. 4A). To investigate the efficacy of AS1892802, fasudil, and tramadol, the maximum doses in MIA experiments were administered. AS1892802 as well as diclofenac showed significant analgesic effects on the mechanical stimulus (Fig. 4B). Tramadol and fasudil were also effective in this model, with efficacies similar to that of AS1892802 (Fig. 4B).

**Site of action**

The tissue distribution of AS1892802 (1 mg/kg) revealed a high plasma concentration in contrast to a low permeability to the brain at 2 h after oral administration (brain/plasma concentration ratio < 0.05, data not shown). In addition, single injection of a very small amount of AS1892802 (3 μg) into the ipsilateral (MIA-treated) knee of MIA rats induced an analgesic effect within 30 min. In contrast, injection of AS1892802 into the contralateral knee had no effect (Fig. 5). These results suggest that AS1892802 induced an analgesic effect mainly through peripheral action.

**Mechanism of action**

To investigate the role of endogenous opioids in the analgesic effect of AS1892802, naloxone, an opioid antagonist (Cox and Welch, 2004), was
injected into AIA rats that had already achieved pain relief by AS1892802 treatment. Although naloxone exclusively antagonized the action of morphine, it did not affect the analgesic activity of AS1892802 (Fig. 6A).

The antinociceptive and anti-inflammatory effects of NSAIDs are understood to be due to the decrease in PGE2 in the inflamed tissue. Although single oral administration of indomethacin (1 mg/kg) strongly reduced the amount of PGE2 in the inflamed paw of AIA rats, AS1892802 (1 mg/kg) did not change PGE2 content (Fig. 6B). Further, AS1892802 did not inhibit human recombinant cyclooxygenase (COX) 1 or 2 protein \textit{in vitro} at up to 10 μM (data not shown). These results suggested that neither endogeneous opioids nor PGE2 are relevant to the analgesic activity of AS1892802. In addition, no improvement was detected in paw swelling of AIA rats even after daily AS1892802 treatment for 6 days (Fig. 6C).

The myosin-binding subunit of myosin light-chain phosphatase has been identified as the substrate of ROCK, and the phosphorylation of the myosin-binding subunit is an essential step in vascular smooth muscle cell contraction (Shimokawa and Rashid, 2007). Given that latrunculin A, an actin-microfilament-disrupting agent, has been reported to cause analgesia on intradermal injection into the inflamed paw of a rat carrageenan model (Dina et al., 2003), we compared the effect of latrunculin A with AS1892802 in AIA rats by intradermal injection. Latrunculin A induced significant pain relief on the inflamed paw of AIA rats as rapidly as did AS1892802 (within 30 min) (Fig. 6D).
The ROCK pathway is well known to control morphological changes, including nerve fiber growth in neuronal cells (Mueller et al., 2005). In our SH-SY5Y human neuroblastoma cell assay, AS1892802 significantly promoted neurite outgrowth at $\geq 10 \text{ nM}$ (Fig. 6E).

**Effect on gastric mucosa, blood pressure, and locomotor activity**

To reveal the safety profile of AS1892802 with chronic dosing, an effect on the gastric mucosa was assessed and compared with NSAIDs. Daily treatment with diclofenac for a week was found to evoke severe ulceration in all rats at 10 mg/kg (Table 1), which is 10 times the minimum effective dose in AIA rats (1 mg/kg, Fig. 4A). Conversely, AS1892802 caused no ulceration at 10 mg/kg (daily treatment for a week), a dose that is more than 30 times the minimum effective dose in MIA rats (0.1 mg/kg, Fig. 3).

ROCK is a major regulator of vascular smooth muscle cells, and ROCK inhibitors are well-known antihypertensive agents (Masumoto et al., 2001). Although AS1892802 also decreased the mean blood pressure in rats, this effect was observed at doses exceeding 10 mg/kg (Fig. 7A).

Tramadol (30 mg/kg) and AS1892802 (1 mg/kg) were effective in both MIA and AIA rats (Fig. 3, 4B). Although tramadol (30 mg/kg) enhanced locomotion in the later phase of locomotor activity assay, AS1892802 administration induced no abnormal behavior at the maximum effective dose (1 mg/kg) in MIA rats (Fig. 7B, C), results which similar to those obtained in a previous study using another
ROCK inhibitor (Inan and Büyükafşar, 2008).
Discussion

**ROCK inhibitor for OA pain**

In the present study, we found that orally administered AS1892802 induced peripheral analgesic effect in rat models of both AIA and MIA. In the case of fasudil, 6 of 27 kinases (including ROCK2) were inhibited more than 50% at 20 μM (Davies et al., 2000). AS1892802 also exerted more than 50% inhibition at 10 μM for 10 of 167 kinases in a kinase panel assay (Supplemental Table 1). However, no kinases were inhibited more strongly than hROCK1, hROCK2, or rROCK2, and only two kinases (PKACα and PRKX) were inhibited more than 80% at 10 μM. The possibility that the analgesic effect of AS1892802 stems from ROCK inhibition is also supported by the following results. First, the plasma concentration of AS1892802 in MIA rats at 2 h after 1 mg/kg injection (285 ng/ml [855 nM]) was 15 times the IC50 value of AS1892802 (57 nM; Fig. 1). Second, no notable metabolites were detected in plasma (data not shown). Third, similar analgesic effects were observed using another ROCK inhibitor with low inhibitory activity against PKACα (IC50 = 1.6 μM: >76-fold selectivity against ROCK) and PRKX (IC50>10 μM: >476-fold selectivity against ROCK) (data not shown).

Anti-inflammatory agents such as NSAIDs are often prescribed for OA pain; however, their effects are limited (Bjordal et al., 2004), and the inflammatory focus is not always detected in the affected joints (Haywood et al., 2003). As such, structural disorders are suspected to cause this non-inflammatory OA
pain (Creamer et al., 1999; Felson et al., 2001). Conversely, discordances between pain intensities and the existence of radiographic changes have been reported in OA patients (Hannan et al., 2000), and up to 40% of patients with severe radiographic changes in joints have been found to not complain of any symptoms (Davis et al., 1992). The trigger of non-inflammatory OA pain is, therefore, still unclear.

In this study, an MIA model was used to predict efficacy of AS1892802 against non-inflammatory OA pain. In the acute phase of this model (Days 1 to 3), inflammatory pathology with limited structural disorder is known to appear after monoiodoacetate injection. In the chronic phase (Days 14 to 28), inflammation is reduced, and severe morphological disorders similar to OA joints are detected (Bove et al., 2003; Fernihough et al., 2004). Diclofenac was found to be effective on pain in the acute phase (data not shown), but not the chronic phase, even at the toxic dose (Fig. 3 and Table 1). Morphological characteristics and response to conventional analgesics suggest similarities between the MIA model and OA patients. AS1892802 is more potent than tramadol, with greater efficacy in an MIA model (Fig. 3), and possesses almost the same efficacy as NSAID in an AIA model (Fig. 4). These results suggest that AS1892802 is a more attractive analgesic than conventional analgesics for the treatment of OA pain.

The maximum efficacy of AS1892802 was superior to that of fasudil (Fig. 3). In this study, analgesic tests were conducted at up to 1 mg/kg (AS1892802) and
30 mg/kg (fasudil), as these compounds caused potent hypotensive effects on normal rats at more than 10 mg/kg (AS1892802, Fig. 7A) and 100 mg/kg (fasudil, data not shown). AS1892802 (1 mg/kg) showed potent analgesic efficacy (full reverse) without inducing critical changes in blood pressure.

AS1892802 exhibited approximately 30-fold higher potency than fasudil in vivo (Fig. 3, ED₅₀: 0.15 mg/kg vs. 4.4 mg/kg) yet almost the same inhibitory activity in vitro (Fig. 2). This discrepancy can be explained by the preferable oral availability of AS1892802. The maximum plasma concentrations of AS1892802 (1 mg/kg) and fasudil (30 mg/kg) were 420 ng/ml (tₘₐₓ= 0.8 h) and 955 ng/ml (tₘₐₓ= 0.5 h), respectively.

**Analgesic mechanism of AS1892802**

Although the role of peripheral ROCK in nociception has not been reported in previous studies, its role in antinociception in central nervous system has been discussed in the following reports. Intrathecal pretreatment of Rho inhibitor BoTX3 or ROCK inhibitor Y-27632 prevented development of allodynia in nerve-injured mice (Inoue et al., 2004). Similarly, intrathecal pretreatment with Y-27632 was also effective against cold allodynia in dorsal rhizotomy rats (Ramer et al., 2004). Further, intrathecal injection of ROCK inhibitor H-1152 caused rapid analgesia on neuropathic pain induced by L5 spinal nerve transection in mice (Tatsumi et al., 2005). Findings from these studies suggest that intrathecally administered ROCK inhibitors relieve neuropathic pain by
reducing demyelination of injured nerves, promoting neuronal plasticity, or reducing nitric oxide synthase activity in the spinal cord. However, these hypotheses cannot be applied to the analgesic mechanism of AS1892802. In the present study, single injection of a small amount of AS1892802 (3 μg) into the knee of MIA rats induced an analgesic effect only on the ipsilateral (MIA-treated) side, and not on the contralateral side (Fig. 5), suggesting that AS1892802 exerts its analgesic effect mainly through peripheral action.

To investigate AS1892802’s mode of action, we first investigated the connection between the compound and endogenous opioids or PGE2. Morphine is a well-known opioid which has a strong analgesic effect in OA patients not only via a systemic route but also via an intra-articular route (Likar et al., 1997). As reported previously (Cox and Welch, 2004), naloxone treatment extinguished most of the analgesic action of morphine but made no notable change on AS1892802 treatment (Fig. 6A). The involvement of ROCK in inflammation has been previously reported (He et al., 2008), and anti-inflammatory agents such as NSAIDs and steroids are known to be effective in relieving inflammatory pain. AS1892802, however, did not alter PGE2 concentration in the inflamed paw of AIA rats (Fig. 6B). Further, AS1892802 induced no decrease in paw volume even at elevated doses in AIA rats (Fig. 6C). In addition, neither AS1892802 nor Y-27632 inhibited lipopolysaccharide-induced tumor necrosis factor (TNF)-α production in human THP-1 monocytic cells (Supplemental Figure 1).
The myosin-binding subunit of myosin light-chain phosphatase has been identified as the substrate of ROCK. Phosphorylation of the substrate through activation of the ROCK pathway is the essential step in vascular smooth muscle cell contraction (Shimokawa and Rashid, 2007). The ROCK-actomyosin pathway is also known to control morphological changes in neuronal cells (Mueller et al., 2005), and AS1892802 (≥ 10 nM) significantly promotes neurite outgrowth in SH-SY5Y human neuroblastoma cells (Fig. 6E), suggesting that AS1892802 plays a role in regulating cytoskeletal function in nerve cells. Latrunculin A, an actin-microfilament-disrupting agent, has been reported to cause analgesia by an intradermal injection into the inflamed paw of a rat carrageenan model (Dina et al., 2003). These results suggest that the intracellular cytoskeleton plays an essential role in the transmission of pain signals. Latrunculin A administered to AIA rats exerted a rapid analgesic effect in the same way as AS1892802 (Fig. 6D), suggesting that AS1892802 induces pain relief via regulation of cytoskeletal function at the peripheral terminal of sensory nerves. Although the precise function of cytoskeleton in ROCK-dependent nociception remains to be elucidated, the present study demonstrated that AS1892802 relieves pain through a mode of action different from that of existing analgesics such as opioids, NSAIDs, and glucocorticoids.

**Safety Profile of ROCK Inhibitor**

The severity of NSAID-induced gastrointestinal toxicity is well known; 8,800 OA
patients are statistically predicted to die from NSAID-induced gastrointestinal toxicity in the United States every year (Singh, 1998), suggesting the narrow safety margin of classical NSAIDs. In our study, severe ulceration of the gastric mucosa was noted on NSAIDs treatment (Table 1); however, repeated administration of AS1892802 for one week resulted in no disorder in gastric mucosa, demonstrating a wider safety margin.

ROCK inhibitors are well-known antihypertensive agents. AS1892802, however, did not indicate significant hypotensive action even up to 3 mg/kg in normotensive rats (Fig. 7A). This wide safety margin may be explained by the characteristics of ROCK inhibitors, which showed potent effects under diseased conditions but not normal conditions (Masumoto et al., 2001).

Tramadol causes central nervous system side effects, including nausea, vomiting, and dizziness with high incidence (Cepeda et al., 2007) and induces abnormal locomotor activity in the range of effective dose (30 mg/kg, Fig. 7B, C). However, AS1892802 did not show any such changes even at the maximum effective dose (1 mg/kg), a potential advantage over tramadol. Taking the present and previous findings into account, AS1892802 seems to have a good safety profile when compared with conventional analgesics for OA pain.

In summary, the novel and highly selective ROCK inhibitor AS1892802 possesses preferable oral availability with markedly low penetration to the brain. AS1892802 showed potent analgesic effects in rat models of both inflammatory (AIA) and non-inflammatory (MIA) pain following therapeutic dosing and
induced none of the critical side effects noted with conventional analgesics. These results suggest that this peripherally-acting ROCK inhibitor may be an attractive analgesic for the treatment of severe OA pain.


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References


Footnotes

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Please send reprint request to: Mr. Eiji Yoshimi
21 Miyukigaoka
Tsukuba-shi, Ibaraki, 305-8585, Japan

E-mail: eiji.yoshimi@jp.astellas.com
**Figure legend**

**Figure 1. Structure of AS1892802.**

**Figure 2. In vitro profile of AS1892802.** (A) Recombinant proteins of human ROCK1, human ROCK2, and rat ROCK2 were used to measure the inhibitory activities of AS1892802 as described in the Methods section. (B) ATP concentration-dependent increase in IC$_{50}$ values. Inhibitory potencies of AS1892802 and fasudil were tested at various ATP concentrations in human recombinant ROCK1 kinase assay. The values are expressed as the mean ± S.E.M. for three experiments.

**Figure 3. Analgesic effect in MIA rats.** AS1892802 (A), fasudil (B), tramadol (C), and diclofenac (D) were administered orally to MIA rats. Two hours later, the analgesic effects were investigated by measuring weight imbalance as described in the Methods section. ED$_{50}$ values were estimated as shown in each figure. (E) Time course of analgesic effects (AS1892802, 1 mg/kg). The values are expressed as the mean ± S.E.M. for eight animals. Significant differences between vehicle and treated groups: *p < 0.05, **p < 0.01; between vehicle and normal groups: &p < 0.05, &&p < 0.01). n.s.= not significant.

**Figure 4. Analgesic effect in AIA rats.** Compounds were administered orally to chronic AIA rats, and 1 h later, the analgesic effects were measured using an
algesimeter as described in the Methods section. (A) diclofenac, (B) AS1892802, fasudil, and tramadol exhibited increases in pain threshold. The values are expressed as the mean ± S.E.M. for 10 animals. Significant differences between vehicle and treated groups: *p < 0.05, **p < 0.01; between vehicle and normal groups: &p < 0.05, &&p < 0.01.

**Figure 5. Intra-articular injection of AS1892802 in MIA rats.** AS1892802 (3 μg) or vehicle was injected into the MIA-treated (ipsilateral) or non-treated (contralateral) knee of chronic MIA rats at a volume of 50 μL at 30 min before the analgesic measurement. The values are expressed as the mean ± S.E.M. for eight animals. Significant differences between vehicle and treated groups: *p < 0.05, **p < 0.01. n.s. = not significant.

**Figure 6. Analgesic mechanism of AS1892802.** (A) AS1892802 (1 mg/kg), Morphine (3 mg/kg), or diclofenac (10 mg/kg) was administered orally to AIA rats. One hour later, the analgesic effects were measured by paw pressure tests as described in the Methods section, and the results are illustrated as “pre-naloxone.” After the first measurement, naloxone (1 mg/kg) was injected subcutaneously, and 5 min later, pain thresholds were determined again (illustrated as “post-naloxone”; N= 8-10). (B) Chronic AIA rats were treated with single oral indomethacin (1 mg/kg) or AS1892802 (1 mg/kg), and the left inflamed hind paws were removed after 1 h. PGE2 concentration in the paw
was measured as described in Methods (N= 5-6). (C) AIA rats were treated with indomethacin (1 mg/kg) or AS1892802 (10 mg/kg) once a day for 6 days, and the volume of left hind paw was measured (N= 5). (D) AS1892802 (3 μg), latrunculin A (500 ng), or vehicle was injected intradermally into the left hind paw of AIA rats. Pain thresholds of the paw were determined at 30 min after the treatment (N= 10). (E) Neurite outgrowth in SH-SY5Y cells. Neurite outgrowth was measured 96 h after AS1892802 treatment was initiated. The values are expressed as mean ± S.E.M. Significant differences between vehicle and treated groups: *p< 0.05, **p< 0.01; between vehicle and normal groups: &p< 0.05, &&p< 0.01

**Figure 7. Blood pressure and locomotor activity.** (A) Time course of changes in mean blood pressure after administration of AS1892802 in conscious rats. Each point with a vertical line represents the mean ± S.E.M. (N= 5-6). (B-C) AS1892802 (1 mg/kg), fasudil (30 mg/kg), or tramadol (30 mg/kg) was administered orally to normal rats. One hour later, locomotor activity was measured for 60 min as described in the Methods section. (B) Total activity for 60 min. (C) Activity every 10 min. The values are expressed as the mean ± S.E.M. for eight animals. Significant differences between vehicle and treated groups: *p< 0.05, **p< 0.01.
Table 1. Effect of AS1892802 on gastric mucosa

<table>
<thead>
<tr>
<th></th>
<th>Dose (mg/kg)</th>
<th>Ulcer incidence (% [n])</th>
<th>Gastric damage score (mm)</th>
<th>Number of ulcers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>0 % [0/5]</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AS1892802</td>
<td>10</td>
<td>0 % [0/5]</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>diclofenac</td>
<td>10</td>
<td>100 % [5/5]</td>
<td>5.6 ± 1.2</td>
<td>2.8 ± 0.5</td>
</tr>
</tbody>
</table>

Gastric damage score and number of ulcers expressed as mean ± S.E.M. for five rats.
Figure 1
Figure 2

A

AS1892802

% of Inhibition

hROCK1

hROCK2

rROCK2

AS1892802 [log M]

B

IC50 (nM)

AS1892802

fasudil

ATP (uM)
Figure 3

A

ED50=0.15 mg/kg

B

ED50=4.4 mg/kg

C

ED50=14 mg/kg

D

n.s.

E

vehicle

AS1892802
Figure 4

A

![Graph showing pain threshold (g) for different doses of diclofenac.](Image)

- Vehicle
- 0.3 mg/kg
- 1 mg/kg
- 3 mg/kg
- 10 mg/kg

B

![Graph showing pain threshold (g) for different drugs and dosages.](Image)

- Normal
- Vehicle
- 1 mg/kg AS1892802
- 30 mg/kg fasudil
- 30 mg/kg tramadol

* p < 0.05
** p < 0.01
Figure 6

A

![Chart showing pain threshold (g) for different treatments](chart)

- Vehicle
- AS1892802
- Morphine
- Diclofenac

Legend:
- Pre-naloxone
- Post-naloxone

B

![Chart showing PGE2 (ng/g tissue)](chart)

- Normal
- Vehicle
- Indomethacin

- Vehicle
- AS1892802
Figure 6

C

D

E

mean neurite length (μm)

0 40 60 80 100 120

0 -9 -8 -7 -6

AS1892802 [log M]

**

vehicle AS1892802

vehicle latrunculin A

**

**

vehicle

paw volume (mL)

normal vehicle indomethacin

normal vehicle AS1892802

**

**

paw volume (mL)

**

vehicle

pain threshold (g)

vehicle AS1892802

vehicle latrunculin A

**

**
Figure 7

A

![Graph showing blood pressure over time with different treatments.]

B

![Bar graph showing locomotor activity with different treatments.]

C

![Graph showing locomotor activity over time with different treatments.]