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R-130823, a novel inhibitor of p38 MAPK, ameliorates hyperalgesia and swelling in arthritis models

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Abstract

We found that a novel compound, R-130823 {2-(4-fluorophenyl)-4-(1-phenethyl-1,2,3,6-tetrahydropyridin-4-yl)-3-(pyridin-4-yl)-1*H*-pyrrole}, had highly selective inhibition against mitogen-activated protein kinase p38α (IC_{50}=22 nM). The release of tumor necrosis factor-α, interleukin-1β, -6 and -8 was inhibited in lipopolysaccharide-stimulated human blood pretreated by R-130823, with IC_{50} values of 0.089, 0.066, 0.95 and 0.16 μM, respectively. R-130823 reduced the established hind paw swelling in rat adjuvant-induced arthritis, while methotrexate showed no suppression. In the same model, R-130823 ameliorated adjuvant-induced hyperalgesia with rapid onset and long duration comparable to a cyclooxygenase-2 inhibitor, celecoxib. In murine collagen-induced arthritis, R-130823 blocked the progress of arthritis when administered just after the onset of the arthritis. Histological analysis of the knee joints showed that proliferation of fibroblasts and synoviocytes and infiltration of neutrophils were ameliorated. In conclusion, R-130823 is expected to be an efficacious treatment for rheumatoid arthritis by blocking the p38 pathway.

Keywords: p38; Tumor necrosis factor-α; Interleukin-1; Adjuvant-induced arthritis; Collagen-induced arthritis

1. Introduction

Rheumatoid arthritis is a chronic and systemic disorder that is characterized by the progressive destruction of articular cartilage and bone. The etiology of rheumatoid arthritis has still not been elucidated but it is thought to be triggered by the combination of genetic susceptibility and exposure to environmental factors (Ollier et al., 2001).

Nowadays, therapeutic strategy focuses on the proinflammatory cytokines that exacerbate the symptoms of rheumatoid arthritis. There are numerous reports that tumor necrosis factor-α, interleukin-1β, -6 and -8 are significantly abundant in joint lesions of rheumatoid arthritis patients (Houssiau et al., 1988; Husby and Williams, 1988; Tetta et al., 1990; Kraan et al., 2001). These cytokines are released from the macrophages that infiltrate joint lesions and the synoviocytes in pannus (Guerne et al., 1989; Yocum et al., 1989; Koch et al., 1991). Proinflammatory cytokines, alone or in combination, induce matrix metalloproteinases which cause destruction of articular cartilage and bone tissues (Ito et al., 1992; Konttinen et al., 1999; Moore et al., 2000). Tumor necrosis factor-α and interleukin-1 also induce cyclooxygenase-2, a regulatory enzyme of prostaglandin synthesis (Jones et al., 1993; Crofford et al., 1994).

Involvement of the proinflammatory cytokines in rheumatoid arthritis is underscored by the findings in recent clinical and preclinical reports of biologic agents. The monoclonal antibody against tumor necrosis factor-α and the tumor necrosis factor-α receptor fusion protein suppress the symptoms and disease progress of rheumatoid arthritis (Elliott et al., 1993; Moreland et al., 1999; Lipsky et al., 2000). Patients treated with the interleukin-1 receptor antagonist showed improvement in clinical studies (Garces,

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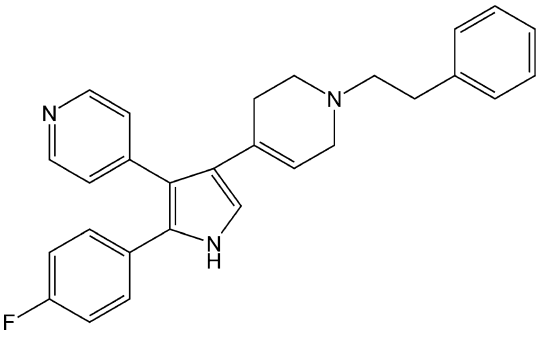

Fig. 1. Chemical structure of R-130823.

2001). Monoclonal antibodies against the interleukin-6 receptor ameliorate collagen-induced arthritis in mice (Takagi et al., 1998) and in monkeys (Mihara et al., 2001).

One of the key factors that regulates expression of the proinflammatory cytokines is serine/threonine protein kinase p38. It belongs to the mitogen-activated protein kinase family and so far four subtypes (p38α, β, γ and δ) have been characterized in mammalian cells (Kyriakis and Avruch, 2001). In response to extracellular stimuli, Thr^{180} and Tyr^{182} of p38α are phosphorylated by upstream mitogen-activated protein kinase kinases MKK3 and MKK6. Activated p38 phosphorylates downstream effectors, e.g., transcription factors and heat shock proteins, and contributes to production of proinflammatory proteins. Several synthetic p38 inhibitors are reported to suppress cytokine production in vitro and show anti-inflammatory effects in acute and chronic inflammation models in vivo (Badger et al., 1996; de Laszlo et al., 1998; Henry et al., 1998; Liverton et al., 1999; Badger et al., 2000; Barone et al., 2001).

In this paper, we describe the anti-inflammatory effects of our novel synthetic compound R-130823 {2-(4-fluorophenyl)-4-(1-phenethyl-1,2,3,6-tetrahydropyridin-4-yl)-3-(pyridin-4-yl)-1*H*-pyrrole}. Inhibition assay of a panel of mitogen-activated protein kinases showed that this compound inhibited p38α, but p38β much less. R-130823 suppressed tumor necrosis factor-α, interleukin-1β, -6 and -8 release from lipopolysaccharide-stimulated human blood. The anti-inflammatory and analgesic effects of R-130823 were confirmed in rat adjuvant-induced arthritis. R-130823 also suppressed the exacerbation of murine collagen-induced arthritis in terms of paw swelling and histopathological analysis of the knee joints. The results obtained so far suggest that R-130823 is a potentially useful agent for the treatment of rheumatoid arthritis.

2. Materials and methods

2.1. Animals

Male Lewis rats (5 weeks old), female Lewis rats (7 weeks old) and male DBA1/J mice (5 weeks old) were obtained from Charles River Japan (Kanagawa, Japan).

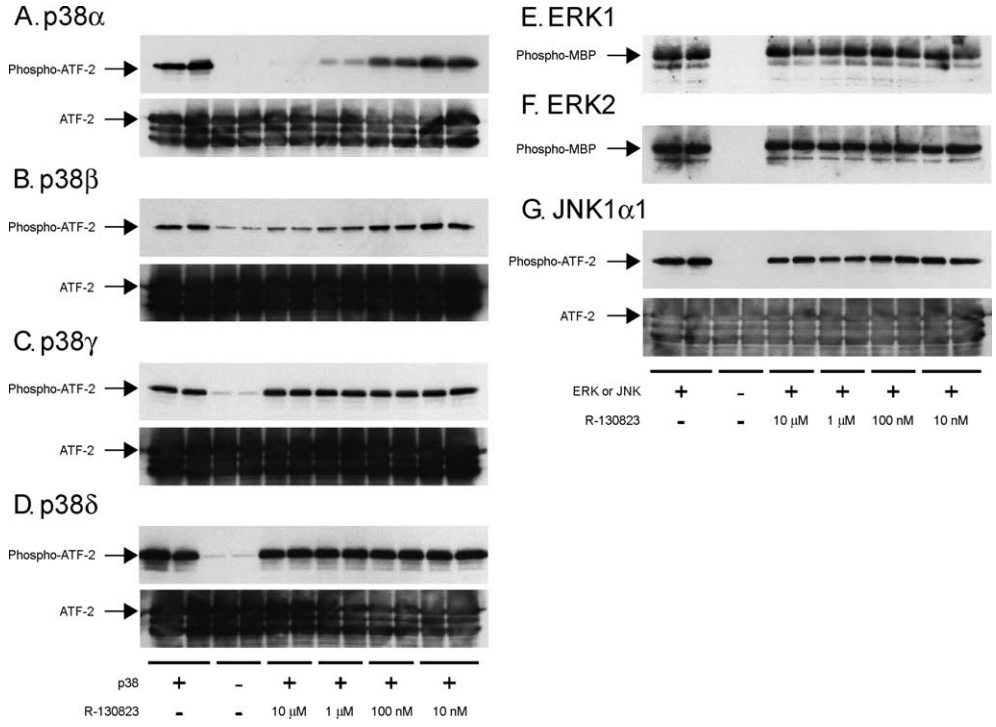

Fig. 2. R-130823 inhibited p38α and p38β but did not inhibit p38γ, p38δ, extracellular signal-regulated kinase 1/2 or c-Jun NH_2-terminal kinase 1α1. Panel A: p38α; Panel B: p38β; Panel C: p38γ; Panel D: p38δ; Panel E: extracellular signal-regulated kinase 1 (ERK1); Panel F: extracellular signal-regulated kinase 2 (ERK2); Panel G: c-Jun NH_2-terminal kinase 1α1 (JNK1α1). Activating transcription factor 2 was incubated with p38α, p38β, p38γ, p38δ, or c-Jun NH_2-terminal kinase 1α1 in the presence of R-130823. Dephosphorylated myelin basic protein was incubated with extracellular signal-regulated kinase 1/2 in the presence of R-130823. Phosphorylation of the substrates was detected by Western blotting. The inhibition of the kinases was assayed in duplicate.

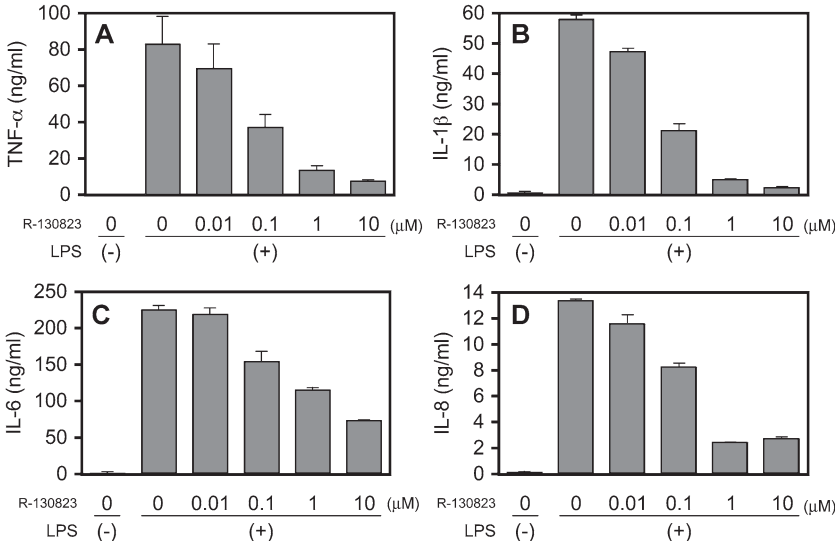

Fig. 3. R-130823 inhibited tumor necrosis factor-α, interleukin-1β, -6 and -8 production in lipopolysaccharide-stimulated human whole blood. Heparinized whole blood was pretreated with R-130823 (0.01–10 μM) 1 h before stimulation with lipopolysaccharide (10 μg/ml) and the cytokine concentrations in the plasma after 6 h-incubation were determined by enzyme-linked immunosorbent assay. Panel A: tumor necrosis factor-α (TNF-α); Panel B: interleukin-1β (IL-1β); Panel C: interleukin-6 (IL-6); Panel D: interleukin-8 (IL-8). The results are expressed as mean inhibition (%) and S.D. of four determinations.

They were fed and given water ad libitum throughout the experimental period unless noted. They were acclimated for about 1 week before use. All animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo.

2.2. Materials

R-130823 {2-(4-fluorophenyl)-4-(1-phenethyl-1,2,3,6-tetrahydropyridin-4-yl)-3-(pyridin-4-yl)-1*H*-pyrrole} and SB 203580 {4-(4-fluorophenyl)-2-(4-methylsulfinyl)-phenyl-5-(pyridin-4-yl)-1*H*-imidazole} were synthesized at the Medicinal Chemistry Research Laboratories, Sankyo (Tokyo, Japan). The structural formula of R-130823 is shown in Fig. 1. Leflunomide {*N*-[4-(trifluoromethyl)-phenyl]-5-methylisoxazole-4-carboxamide} and celecoxib {5-(4-methylphenyl)-1-(4-sulfamoylphenyl)-3-trifluoro-methyl-1*H*-pyrazole} were synthesized at Chemtech Labo. (Tokyo, Japan). Methotrexate and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO). Lipopoly-saccharide (*E. coli* 026:B6) and *Mycobacterium butyricum* were purchased from BD Diagnostic Systems (Sparks, MD). Bovine type II collagen solution was purchased from the Collagen Research Center (Tokyo, Japan). Activated human recombinant p38α, p38β, p38γ, p38δ, extracellular signal-regulated kinase 1, c-Jun NH_2-terminal kinase 1α1 and activated mouse recombinant extracellular signal-regulated kinase 2 were purchased from Upstate Biotechnology (Lake Placid, NY).

2.3. p38 and c-Jun NH_2-terminal kinase inhibition assay

Inhibitory activities on p38 and c-Jun NH_2-terminal kinase 1α1 were measured using p38 MAP kinase assay kit and anti-activating transcription factor 2 antibody (both from Cell Signaling Technology, Beverly, MA). Briefly, activated p38 and c-Jun NH_2-terminal kinase 1α1 were diluted in kinase buffer at 1.25 μg/ml (p38α and p38γ), 0.625 μg/ml (p38β), 0.313 μg/ml (p38δ) or 37.5 mU/ml (c-Jun NH_2-terminal kinase 1α1). In microtubes, a 20-μl aliquot of the kinase solution was mixed with 20 μl of a test compound (diluted in 0.3% dimethyl sulfoxide/kinase buffer), 10 μl of 1.2 mM ATP and 10 μl of 400 μg/ml activating transcription factor 2 fusion protein.

Table 1
IC_{50} values of R-130823 and SB 203580 on cytokine production in lipopolysaccharide-stimulated human whole blood

Compound	IC_{50} (μM)			
	Tumor necrosis factor-α	Interleukin-1β	Interleukin-6	Interleukin-8
R-130823	0.089 (0.056–0.14)	0.066 (0.054–0.080)	0.95 (0.75–1.2)	0.16 (0.13–0.21)
SB 203580	5.2 (4.7–5.8)	0.43 (0.22–0.77)	>10	1.2 (0.86–1.7)

Heparinized whole blood was pretreated with the test compounds 1 h before stimulation by lipopolysaccharide (10 μg/ml). Tumor necrosis factor-α, interleukin-1β, -6 and -8 concentrations in the plasma after 6-h incubation were determined by enzyme-linked immunosorbent assay. Results are given as mean and S.D. of three to four determinations.

The microtubes were incubated at 30 °C for 30 min. The tubes were then chilled at 4 °C and 60 μl of sodium dodecyl sulfate sample buffer was immediately added. The tubes were boiled in a water bath for 5 min and centrifuged at 15,000×*g* at 4 °C for 2 min. A 20-μl aliquot was subjected to 4–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Daiichi Kagaku, Tokyo, Japan) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Phosphorylated and total activating transcription factor 2 were detected with Phototope®-HRP Western Detection Kit (Cell Signaling Technology). Optical density of bands was analyzed by NIH Image (Ver. 1.62; the National Institute of Mental Health, Rockville, MD).

IC_{50} values were calculated based on the regression line defined below:

$$\text{Inhibition } (\%) = a + b \times \text{Log } x,$$

where a and b are unknown parameters and x is the concentration of the compound. The point estimate of IC_{50} was calculated as follows:

$$IC_{50} = 10^{(50-a)/b}$$

The 95% confidence intervals were calculated by Fieller's theorem.

2.4. Extracellular signal-regulated kinase inhibition assay

Inhibition activities on extracellular signal-regulated kinase 1/2 were measured using MAP Kinase Assay Kit (Upstate Biotechnology). Briefly, activated extracellular signal-regulated kinases were diluted in kinase buffer at 50 μg/ml (extracellular signal-regulated kinase 1) or 5 μg/ml (extracellular signal-regulated kinase 2). In microtubes a 10-μl aliquot was mixed with 10 μl of the assay dilution buffer from the kit, 20 μl of a test compound (diluted in 0.3% dimethyl sulfoxide/kinase buffer), 10 μl of 75 mM $MgCl_2$/500 μM ATP and 10 μl of 2 mg/ml dephosphorylated myelin basic protein solution.

The microtubes were incubated at 30 °C for 30 min and then chilled at 4 °C. A 30-μl aliquot was immediately transferred to another tube to which was added 70 μl of chilled phosphate-buffered saline. Then, 100 μl of sodium dodecyl sulfate sample buffer was added and the tube was boiled for 5 min. After centrifuging at 15,000×*g* at 4 °C for 2 min, a 20-μl aliquot was subjected to 10–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Phosphorylated myelin basic protein was detected with anti-phospho-myelin basic protein antibody, along with anti-mouse IgG horseradish peroxidase-linked antibody and LumiGLO™ Reagent and Peroxide (both from Cell Signaling Technology).

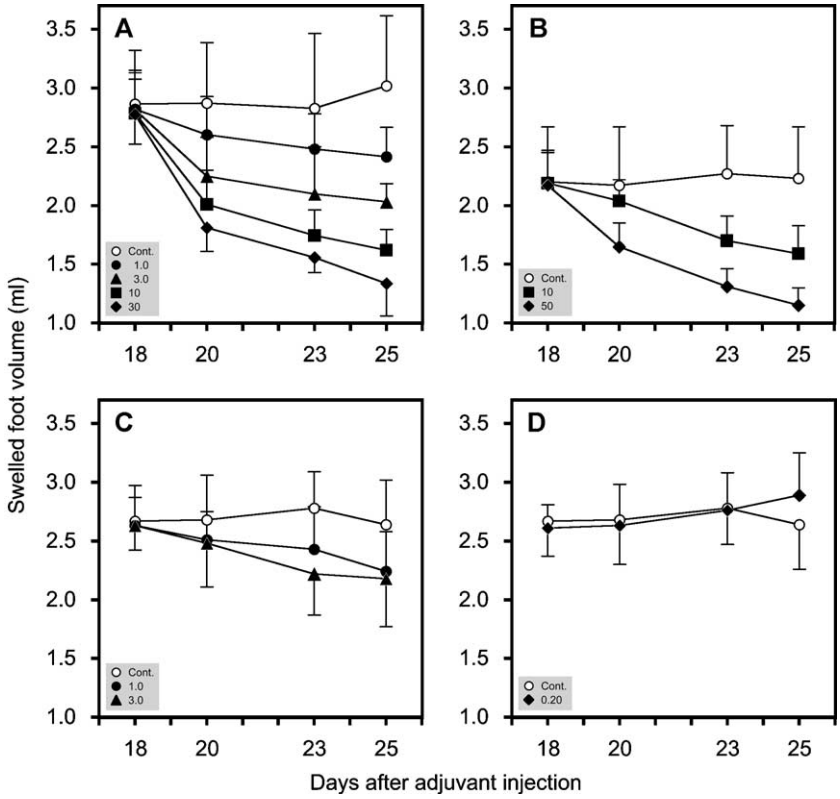

Fig. 4. Therapeutic effect of R-130823 on rat adjuvant-induced arthritis. *M. butyricum* was injected intradermally into the heel of the right hind footpad on Day 0. Test compounds were administered twice a day from Day 18 to Day 24. Panel A: R-130823 (1.0, 3.0, 10, 30 mg/kg/day); Panel B: SB 203580 (10, 50 mg/kg/day); Panel C: leflunomide (1.0, 3.0 mg/kg/day); Panel D: methotrexate (0.20 mg/kg/day). Results are shown as mean and S.D. of swelled foot volumes of five to eight animals in each group.

2.5. Cell-based assay of cytokine release inhibition

Fresh blood was collected aseptically in the presence of heparin by venipuncture from healthy adult volunteers. A 988-μl aliquot of blood was mixed with 2 μl of either a test compound solution or dimethyl sulfoxide and 10 μl of 1.0 mg/ml lipopolysaccharide (dissolved in phosphate-buffered saline; final concentration of 10 μg/ml) in microtubes. The test compounds dissolved in dimethyl sulfoxide were used immediately. The blood mixture was incubated at 37 °C for 6 h, immediately chilled at 4 °C and then centrifuged at 15,300×*g* for 5 min. The plasma was stored at −20 °C until use. Concentrations of tumor necrosis factor-α, interleukin-1β, -6 and -8 in the plasma were determined by enzyme-linked immunosorbent assays (Bio-Source International, Camarillo, CA).

IC_{50} values were calculated based on linear regression lines obtained from the percent inhibition and the logarithmic values of the doses. The 95% confidence intervals were calculated by Fieller's theorem.

2.6. Therapeutic experiment on established adjuvant-induced arthritis

The experiment was performed according to the method described by Winder et al. (1969) with some modifications. Briefly, an adjuvant was prepared by suspending heat-killed dried *M. butyricum* in dry-sterilized liquid paraffin (Wako, Osaka, Japan) to make a 4.0 mg/ml suspension and sonicated with Sonifier® Cell Disruptor 200 (Branson Ultrasonics, Danbury, CT). The adjuvant (200 μg/0.05 ml/paw) was injected intradermally into the heel of the right hind footpad of female Lewis rats on Day 0. On Day 18, when the swelled foot volume almost reached a plateau in the majority of the animals, the animals with prominent swelling in the adjuvant-injected foot were selected. The animals were divided into groups so that the mean swelled foot volumes were equivalent. Test compounds were suspended in 0.5% sodium carboxymethyl cellulose (Daiichi Pure Chemicals) and orally administered in a volume of

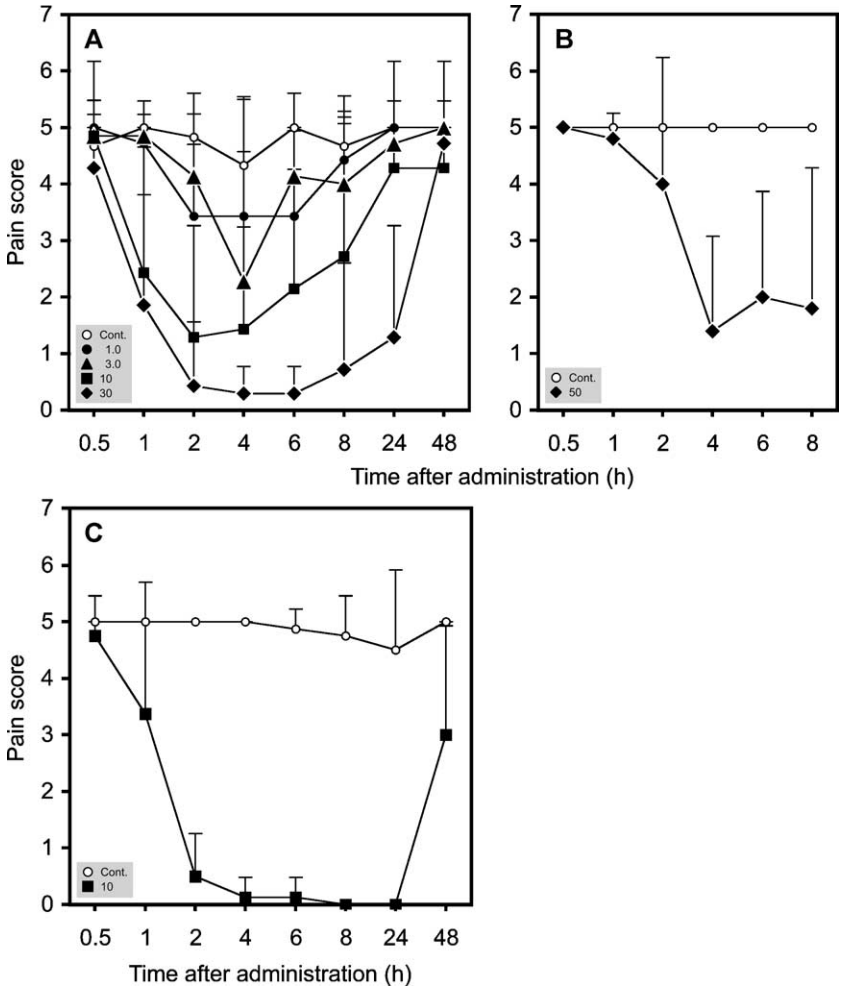

Fig. 5. Analgesic effect of R-130823 on chronic inflammatory pain in rat adjuvant-induced arthritis. *M. butyricum* was injected intradermally into the heel of the right hind footpad on Day 0. On Day 18, after the test compounds were administered, the ankle joint of the left foot was flexed five times at each measurement point. The pain score (the number of squeaks) was examined by an independent observer in a blind manner. Panel A: R-130823 at 1.0 (●), 3.0 (▲), 10 (■) and 30 mg/kg/day (◆), respectively and vehicle control (○); Panel B: SB 203580 at 50 mg/kg (◆) and vehicle control (○); Panel C: celecoxib at 10 mg/kg (■) and vehicle control (○). Results are shown as mean and S.D. of the pain scores of five to eight animals in each group.

2 ml/kg twice a day from Day 18 to Day 24. The volume of the adjuvant-injected foot was measured on Days 18, 20, 23 and 25 by a plethysmometer (Ugo Basile, Italy).

2.7. Analgesic effect on adjuvant-induced arthritic pain

The experiment was performed according to the method by Kuzuna and Kawai (1975) with some modifications. The adjuvant containing *M. butyricum* was prepared as described in the previous section. It was injected intradermally into the heel of the right hind footpad of male Lewis rats (200 μg/0.05 ml/paw) on Day 0. On Day 17 the animals, whose arthritis had been well established, were fasted overnight. The next day, pain-positive rats were selected by the response to gentle flexing of the tarso-tibial joint of the left foot for five times. An animal was defined as pain-positive when it squeaked at every flexion. The pain-positive rats were randomly divided into groups and were administered orally with test compounds suspended in 0.5% sodium carboxymethyl cellulose. The pain response was examined in a blind manner in animals at 0.5, 1, 2, 4, 6, 8 h after the administration by an independent observer. In the R-130823 and celecoxib-administered groups, the pain responses at 24 and 48 h were also examined. The pain score was defined as the number of squeaks. The pain score was subjected to repeated measures analysis of variance, followed by non-parametric Dunnett's test for the comparison between each dose and the control group.

2.8. Collagen-induced arthritis

Bovine type II collagen solution was mixed with 0.01 M acetic acid and Freund's complete adjuvant (BD Diagnostic Systems) in a 2:1:3 ratio by POLYTRON® (KINEMATICA, Switzerland) to make 1 mg/ml type II collagen emulsion. DBA1/J mice were injected intradermally with 0.1-ml emulsion at the base of the tail. Twenty-two days after this, the mice received a booster injection as well. The mice were assessed daily by the following grades: 0=absence of arthritis; 1=swelling of one toe; 2=swelling of two or more toes; 3=swelling of tarsus and ankle; and 4=severe swelling or bony deformity. For each mouse, the day when arthritis was first observed (grade≥3) on either hind paw was designated as Day 0. From that day the mouse was orally administered once daily R-130823 for 2 weeks or dexamethasone for 1 week. All paws were examined daily and the arthritis index was calculated as the sum of the grades (maximal index=16 per animal).

The arthritis index was subjected to repeated measures analysis of variance with the index on Day 0 as a covariate. Individual comparisons were made between each treatment and the control group using Dunnett's test.

The animals in the R-130823 groups were sacrificed after 2 weeks of administration. The knee joints from the hind limbs of which the paw had a score of 3 or more on Day 0 were fixed in 10% buffered neutral formalin solution. After trimming the region of the knee joint in the sagittal section, the specimens were decalcified with 10% ethylenediaminetetraacetic acid. Block specimens were prepared in a conventional manner, thin-sectioned and stained with hematoxylin and eosin, and safranin O. The specimens were examined by an independent observer in a blind manner. The bone and synovial membrane of the knee joints were evaluated using the specimens stained with hematoxylin and eosin. The cartilage was observed using the specimens stained with safranin O. Specimens were graded as follows: 0=normal; 1=mild; 2=moderate; and 3=severe.

3. Results

3.1. Mitogen-activated protein kinase inhibition assay

We evaluated R-130823 (Fig. 1) in terms of the inhibition of p38 and other mitogen-activated protein kinases. R-130823 was identified as inhibiting p38α in a concentration-dependent manner (Fig. 2). It also showed moderate inhibitory activity on p38β. The IC_{50} value was 22 nM

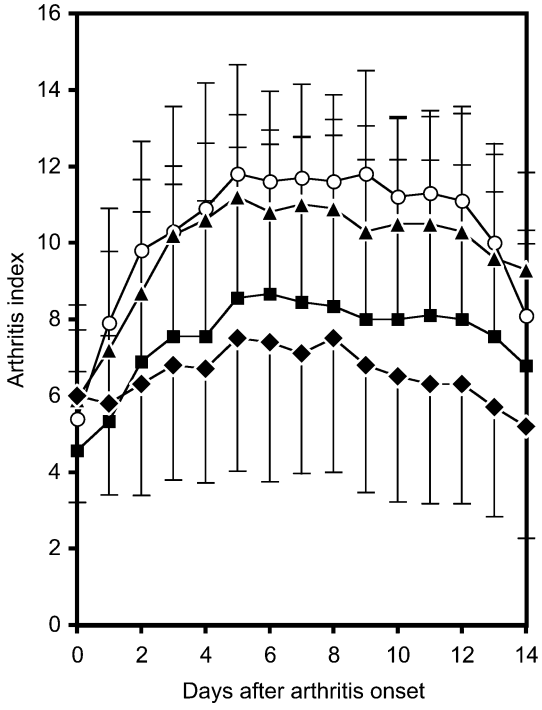

Fig. 6. Suppression of exacerbation of collagen-induced arthritis by R-130823 in DBA1/J mice. DBA1/J mice were injected and boosted with type II collagen emulsion. For each mouse, the day when it elicited arthritis (grade≥3) on either hind paw was designated as Day 0. From that day the mouse was orally administered R-130823 once daily for 2 weeks. All paws were examined daily and the arthritis index was calculated as the sum of the grades. Symbols are 3.0 (▲), 10 (■), 30 mg/kg/day (♦) of R-130823 and vehicle control (○). The arthritis index at 30 mg/kg/day was significantly different from that of the vehicle control ($P<0.0001$ by Dunnett's test). Results are shown as mean and S.D. of arthritis indices of 9–10 animals in each group.

(95% confidence interval: 5.0–58 nM) for p38α and 820 nM (360–2.4×10^3 nM) for p38β. Neither p38γ, p38δ, extracellular signal-regulated kinase 1/2 nor c-Jun NH_2-terminal kinase 1α was inhibited at up to 10 μM.

3.2. Cell-based assay of cytokine release inhibition

We then studied whether R-130823 inhibited cytokine release from human whole blood stimulated with lipopolysaccharide. Human blood was pretreated with the test compounds for 1 h and then stimulated with lipopolysaccharide. After incubation for 6 h, cytokines in the plasma were analyzed by enzyme-linked immunosorbent assay. R-130823 inhibited tumor necrosis factor-α, interleukin-1β, -6 and -8 with IC_{50} values of 0.089 μM (95% confidence interval: 0.056–0.14 μM), 0.066 μM (0.054–0.080 μM), 0.95 μM (0.75–1.2 μM) and 0.16 μM (0.13–0.21 μM), respectively (Fig. 3 and Table 1). A reference p38 inhibitor, SB 203580, also inhibited these cytokines, although its IC_{50} value for interleukin-6 was greater than 10 μM.

3.3. Therapeutic effect on adjuvant-induced arthritis in rats

In this arthritis model, chronic inflammatory swelling was elicited in the hind paw of animals 18 days after adjuvant injection. To investigate whether R-130823 had any therapeutic effect on the established arthritis, R-130823 was administered to the rats twice a day at total daily doses of 1, 3, 10 and 30 mg/kg from Day 18 to Day 24. R-130823 produced dose-dependent inhibition of the swelling (Fig. 4A). On Day 25, R-130823 suppressed the swelling volume by 18%, 31%, 45% and 55% at 1, 3, 10 and 30 mg/kg, respectively. On the same day (Day 25), SB 203580 suppressed the swelling volume by 28.6% and 48.2% at 10 and 50 mg/kg, respectively (Fig. 4B). The anti-rheumatic drug leflunomide, which was administered at 1 and 3 mg/kg, showed potency comparable to R-130823 of the same doses (Fig. 4C). On the other hand, methotrexate, another popular agent for rheumatoid arthritis, hardly affected the swelling (Fig. 4D) at the dose which exerts high potency in a prophylactic regimen (Jaffee et al., 1989).

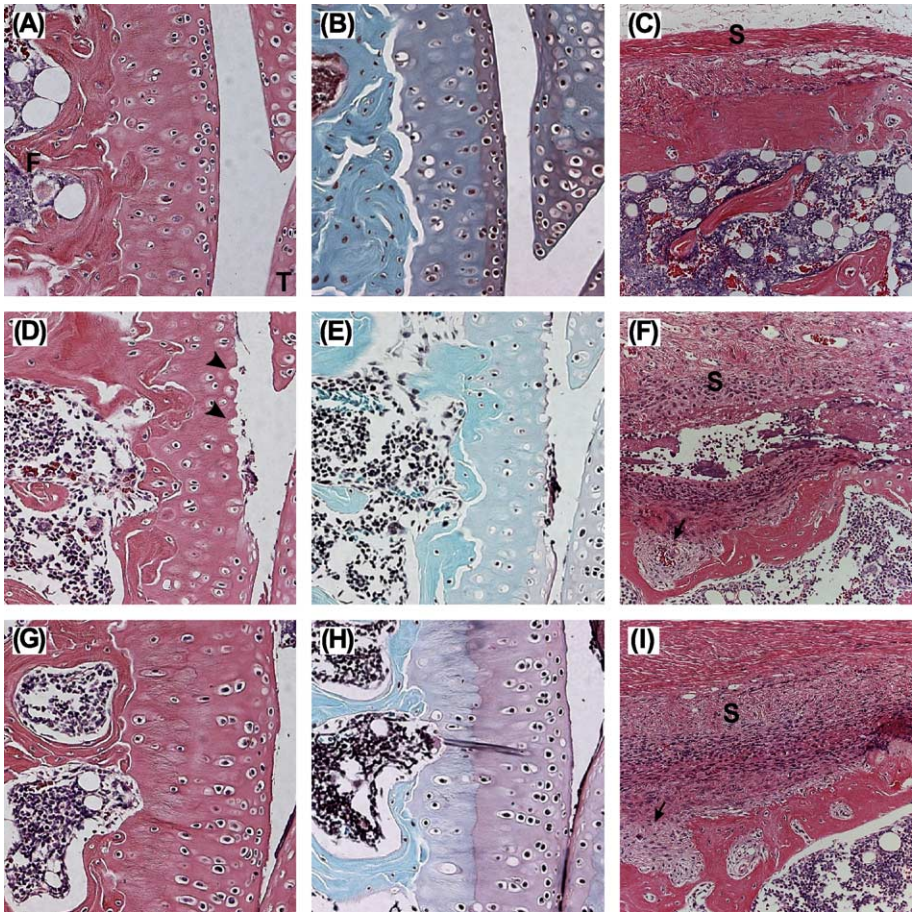

Fig. 7. Microphotographs of representative hind knee joints of collagen-induced arthritis. Panels A–C: normal joints from non-immunized mice. The surface of articular cartilage (A, B) and synovium (C) are shown. Panels D–F: joints from collagen-induced arthritis mice treated with the vehicle control. Note the degeneration (arrowhead) of cartilage cells (D), and proliferation of synovial cells and fibroblasts that destroyed bone tissues (F; arrow). Panels G–I: joints from collagen-induced arthritis mice administered R-130823 (30 mg/kg/day). The cartilage surface has been maintained (G, H), but destruction of bone tissue is still seen (I; arrow). F, femur; T, tibia; S, synovium. Panels A, C, D, F, G and I were stained with hematoxylin and eosin. Panels B, E and H were stained with safranin O. See Materials and methods for details.

3.4. Analgesic effect on adjuvant-induced arthritic pain in rats

The analgesic effect of R-130823 on chronic inflammatory pain was evaluated in the adjuvant-induced arthritis model. In this assay, rats responded to the flexing of swelled ankle joints with squeaks, the number of which was defined as the pain score. After R-130823 was administered, the pain score was decreased in a dose-dependent manner. It decreased quickly within 1 h after the administration and the maximum effect was observed 2 h later (Fig. 5A). At 30 mg/kg, R-130823 retained analgesic effect until 24 h after administration ($P<0.05$ during 1–24 h). A 74% decrease in the pain score was still observed at 24 h. We also evaluated the analgesic effect of SB 203580. SB 203580 of 50 mg/kg was administered to the arthritic rats and it started to decrease the pain score 2 h later with the maximum effect observed 4 h later (Fig. 5B).

As a positive control, a nonsteroidal anti-inflammatory drug, celecoxib, was administered. Celecoxib of 10 mg/kg began to reduce the pain score 1 h later (Fig. 5C). At 2 h, celecoxib showed maximum effect, which was sustained up to 24 h. The analgesic effect diminished 48 h after the administration.

3.5. Collagen-induced arthritis

We tested R-130823 in murine collagen-induced arthritis, another rheumatoid arthritis model. R-130823 was administered to DBA1/J mice after the paw swelling was evident. R-130823 suppressed the progress of arthritis in a dose-dependent manner from 3 to 30 mg/kg and the animals treated with 30 mg/kg showed no increase in the arthritis index (Fig. 6), which was lower than the vehicle control group with statistical significance ($P<0.0001$). As a positive control, we tested the effects of dexamethasone on this arthritis model. Dexamethasone at 0.3 mg/kg reduced the arthritis index to zero after 4 days of administration (data not shown).

In order to investigate the effects of R-130823 on the destruction of joint tissues, the hind knee joints of the mice were histologically examined the day after the last day of the 2-week administration period. Hematoxylin and eosin staining of the joints of normal mice showed structural integrity of cartilage and bone tissues (Fig. 7A and C). Normal condition of the cartilage was also confirmed by safranin O staining (Fig. 7B). In the vehicle control group, chondrocytes showed degenerative and necrotic conditions (Fig. 7D). Infiltration of neutrophils was observed in the synovial membrane and the proliferation of synoviocytes and fibroblasts led to destruction of cartilage and bone tissues (Fig. 7F). Debris and deposition of fibrin were found in the joint cavity.

In the R-130823-treated group (30 mg/kg/day), inhibitory effect on the desquamation of cartilage cells was observed (Fig. 7G and H). Infiltration of neutrophils and the proliferation of fibroblasts and synoviocytes were milder than those of the control, while destruction of cartilage and bone tissues was not affected (Fig. 7I).

These histological conditions were graded by an independent observer in a blind manner (Table 2). Infiltration of neutrophils, deposition of fibrin, the proliferation of synovial cells and fibroblasts, the presence of debris and desquamation of cartilage cells were less severe than those of the control. In contrast, the degree of these symptoms at the doses of 3 and 10 mg/kg/day were nearly the same as those of the control (data not shown).

Table 2
Histological analysis of the effect of R-130823 treatment on collagen-induced arthritis

Observation item	Immunization:	–	+	
	Treatment:	–	Vehicle	R-130823 (30 mg/kg)
Synovial membrane				
Infiltration of neutrophils		0.00±0.00	2.67±0.58	1.33±1.53
Deposition of fibrin		0.00±0.00	1.00±0.00	0.67±0.58
Proliferation of synovial cells		0.00±0.00	2.00±0.00	0.33±0.58
Proliferation of fibroblasts		0.00±0.00	2.33±0.58	1.67±0.58
Presence of debris in the cavity		0.00±0.00	2.00±0.00	1.00±1.00
Cartilage tissues				
Degeneration and/or necrosis of cartilage cells		0.00±0.00	1.00±0.00	1.00±1.00
Desquamation of cartilage cells		0.00±0.00	1.00±0.00	0.00±0.00
Destruction of cartilage tissues		0.00±0.00	1.00±0.00	1.00±1.00
Bone tissues				
Destruction of bone tissues		0.00±0.00	1.00±0.00	1.00±1.00

DBA1/J mice were immunized and boosted with bovine type II collagen. For each mouse, the day when arthritis was first observed (grade≥3) on either hind paw was designated as Day 0. From that day the mouse was administered R-130823 once daily for 2 weeks. After this period, the hind knee joints of the animal whose paw on the same limb had been graded ≥3 on Day 0 were fixed in 10% buffered neutral formalin solution. An independent observer evaluated the bone and synovial membrane using the specimen stained with hematoxylin and eosin. The cartilage was observed using the specimens stained with safranin O. The symptoms were graded as follows; 0 for normal, 1 for mild, 2 for moderate and 3 for severe. Results are shown as mean and S.D. of the grades of three animals in each group.

4. Discussion

A novel compound, R-130823, was found to inhibit p38α and p38β in a mitogen-activated protein kinase inhibition assay. Considering that the IC_{50} for p38α (22 nM) is 37-fold less than that for p38β (820 nM), R-130823 is essentially specific for the p38α isoform. In addition, R-130823 did not inhibit p38 isoforms γ or δ, or other mitogen-activated protein kinase family members such as extracellular signal-regulated kinases and c-Jun NH_2-terminal kinase 1 up to 10 μM. This specificity is in common with p38 inhibitors SB 203580 (Lee et al., 1999) and L-167307 (de Laszlo et al., 1998).

Crystallographic analysis of the complex of p38α and pyridinyl-imidazole inhibitors demonstrated that the inhibitors compete with ATP at the ATP-binding site (Tong et al., 1997; Wilson et al., 1997). Structural similarity between the compounds and R-130823 strongly suggests that R-130823 inhibits via the same mechanism, although it remains to be confirmed.

We then carried out a lipopolysaccharide-stimulated human whole blood assay to evaluate the anti-inflammatory effects of R-130823 in vitro. Lipopolysaccharide is a principal component of gram-negative bacteria (Morrison and Ryan, 1987) and activates the p38 pathway (Raingeaud et al., 1996; Lee et al., 2000). p38 regulates mRNA of tumor necrosis factor-α (Wang et al., 1999), interleukin-1 (Caivano and Cohen, 2000), interleukin-6 (Wang et al., 1999) and interleukin-8 (Manthey et al., 1998).

We used whole blood instead of purified monocytes for the assay since it is closer to physiological conditions. R-130823 inhibited tumor necrosis factor-α as well as interleukin-1, -6 and -8 release from human whole blood. R-130823 showed well-balanced inhibition against the release of all the cytokines investigated, while SB 203580 inhibited interleukin-6 more weakly than the other cytokines. This profile is expected to be effective in inflammatory diseases such as rheumatoid arthritis where multiple cytokines are simultaneously produced (Eastgate et al., 1988; Houssiau et al., 1988; Husby and Williams, 1988; Tetta et al., 1990; Kraan et al., 2001).

Rat adjuvant-induced arthritis is a commonly used animal model for preclinical studies of nonsteroidal anti-inflammatory drugs and disease-modifying anti-rheumatic drugs (Billingham, 1983). When R-130823 was administered in a therapeutic protocol, dose-dependent decrease in the hind paw swelling was observed. To our knowledge, there are no studies so far that have identified the p38 isoform involved in the development of the arthritis. However, considering that p38α is widely expressed in inflammatory cells such as monocytes and is more activated by exogenous stimuli than other p38 isoforms (Hale et al., 1999), R-130823 most likely inhibited p38α in inflammatory lesions. Since tumor necrosis factor-α, interleukin-1 and -6 concentrations are elevated in joints in which arthritis has developed (Leisten et al., 1990; Smith-Oliver et al., 1993; Silva et al., 2000), anti-inflammatory effects of R-130823 can be explained partly by the suppression of these cytokines. Another possible mechanism of the effects of R-130823 is suppression of cyclooxygenase-2 expression. Cyclooxygenase-2 is induced in response to cytokine stimulation (O'Banion et al., 1992; Crofford et al., 1994; Amin et al., 1999) and regulates prostaglandin synthesis in inflammatory sites. p38 is involved in the expression of cyclooxygenase-2 at both transcriptional and posttranscriptional levels in synovial fibroblasts (Faour et al., 2001). Besides, we observed that R-130823 suppressed interleukin-1-induced cyclooxygenase-2 expression in chondrocytes (paper in submission).

Methotrexate is an acknowledged disease-modifying drug for rheumatoid arthritis. Although it is not fully elucidated, the primary target of methotrexate seems to be a folate-dependent enzyme, not the cytokine synthesis pathway (Andersson et al., 2000). In our study, therapeutic use of methotrexate did not have any significant effect on the established stage of adjuvant-induced arthritis. As discussed by Sakuma et al. (2001), anti-folate action of methotrexate may not be effective on inflammatory cells which have already infiltrated the lesions. In other words, R-130823, by inhibition of cytokine synthesis, is expected to have efficacy even in patients whose arthritis resists methotrexate treatment. In fact, etanercept, a soluble tumor necrosis factor receptor fusion protein, improved the symptoms in rheumatoid arthritis which were persistent despite receiving methotrexate (Weinblatt et al., 1999).

The analgesic effect of R-130823 was studied on rat adjuvant arthritis. R-130823 ameliorated adjuvant-induced hyperalgesia in a dose-dependent manner. Analgesic effect was observed as well in the SB 203580-administered group. As far as we know, this is the first report demonstrating that p38 inhibitors exert analgesic action on chronic pain in arthritis models. R-130823 suppressed the pain score 24 h after administration, with similar kinetics to celecoxib. While the cyclooxygenase-2 inhibitor celecoxib inhibits cyclooxygenase-2 activity, R-130823 probably down-regulates cyclooxygenase-2 expression as discussed above, resulting in the similarity of the kinetics between these two compounds. p38 also mediates hyperalgesia through the bradykinin B_1 receptor in adjuvant-induced arthritis (Ganju et al., 2001). R-130823 might, therefore, interfere with the bradykinin-induced signal transduction. Although it requires further study to determine which factor is predominant, the results imply that R-130823 simultaneously provides anti-inflammatory and analgesic actions in rheumatoid arthritis treatment.

Collagen-induced arthritis in DBA/1 mice is an arthritis model in which synovial infiltration and joint destruction similar to those of human rheumatoid arthritis are observed (Stuart et al., 1982). We tested the therapeutic effects of R-130823 in the animals with severe symptoms (grade≥3), because efficacy on established arthritis is clinically more relevant than prophylactic treatment. During the 2 weeks of

the administration period, R-130823 prevented progress of the arthritis. Involvement of proinflammatory cytokines in collagen-induced arthritis has been reported in previous studies using transgenic mice deficient in tumor necrosis factor or interleukin-6 (Alonzi et al., 1998; Sasai et al., 1999; Campbell et al., 2001), or anti-interleukin-1 receptor or anti-tumor necrosis factor-α antibodies (Williams et al., 2000). Considering that R-130823 suppressed all of tumor necrosis factor-α, interleukin-1 and -6 in vitro, concomitant suppression of these cytokines may have led to this complete block of the arthritis progress.

Histological analysis showed that R-130823 ameliorated synovial hyperplasia, neutrophil infiltration and desquamation of chondrocytes, even after the arthritis had been established. Meanwhile, persistent symptoms in bone and cartilage tissues underscored the irreversible process of joint destruction in the arthritis model. Involvement of tumor necrosis factor-α and interleukin-1 was suggested in reports of the treatment of established murine arthritis with both neutralizing antibodies against and soluble receptors to tumor necrosis factor-α or interleukin-1 (Williams et al., 1992; Joosten et al., 1999; Bessis et al., 2000). In a clinical study, infliximab (anti-tumor necrosis-α antibody) treatment reduced the progress of joint damage in rheumatoid arthritis patients (Lipsky et al., 2000). Since tumor necrosis factor-α and interleukin-1 are simultaneously suppressed, it is expected that R-130823 protects histological integrity in rheumatoid arthritis.

In summary, we have shown R-130823 inhibited p38 mitogen-activated protein kinase. It was selective to the α isoform and, less potently, to the β isoform. R-130823 significantly reduced lipopolysaccharide-induced cytokine release in human whole blood. It demonstrated therapeutic and analgesic effects on animal arthritic models. Thus, R-130823 is expected to be a candidate as a new anti-inflammatory agent for the treatment of rheumatoid arthritis.

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