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Prevention of progressive joint destruction in collagen-induced arthritis in rats by a novel matrix metalloproteinase inhibitor, FR255031

*,[1]Takeshi Ishikawa, [1]Fusako Nishigaki, [1]Susumu Miyata, [1]Yoshitaka Hirayama, [1]Kyoko Minoura, [1]Junko Imanishi, [2]Masahiro Neya, [2]Tsuyoshi Mizutani, [3]Yoshimasa Imamura, [4]Yoichi Naritomi, [4]Hidetsugu Murai, [1]Yoshitaka Ohkubo, [4]Akira Kagayama & [1]Seitaro Mutoh

[1]Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd, 2-1-6, Kashima, Yodogawa-ku, Osaka 532-8514, Japan; [2]Medicinal Chemistry Research Laboratories, Fujisawa Pharmaceutical Co., Ltd, Ibaraki, Japan; [3]Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd, Ibaraki, Japan and [4]Biopharmaceutical and Pharmacokinetic Research Laboratories, Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan

1 FR255031 (2-[(7*S*)-7-[5-(4-ethylphenyl)-2-thienyl]-1,1-dioxido-4-(2-pyridinylcarbonyl)hexahydro-1,4-thiazepin-7-yl]-*N*-hydroxyacetamide) is a novel synthetic matrix metalloproteinase (MMP) inhibitor that inhibits human collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9) and membrane type 1 MMP (MT1-MMP/MMP-14). FR255031 also inhibits rat collagenase and gelatinase. We studied the effect of FR255031 and Trocade, an inhibitor of collagenase and MMP-14, on a rat collagen-induced arthritis (CIA) model.

2 Rat CIA was induced by intradermal injection of type II collagen (IIC) and oral administration of FR255031 or Trocade was performed for 28 days. Body weight loss, hind paw swelling, elevation of serum anti-IIC antibody, and histological and radiographic scores were evaluated.

3 FR255031 markedly inhibited cartilage degradation in a dose-dependent manner in the CIA model, but Trocade failed to prevent the degradation.

4 FR255031 at a dose of 100 mg kg^{-1} also had statistically significant effects on bone destruction and pannus formation and on the recovery of body weight loss on day 28.

5 These results indicate that FR255031 is effective for rat CIA, especially on joint cartilage destruction. These data suggest that as well as collagenases or MT-MMP, gelatinases are also involved in joint destruction in arthritis.

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Keywords: Matrix metalloproteinase inhibitor; collagenase; gelatinase; collagen-induced arthritis; pharmacokinetics

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin repeats; AUC, area under the plasma concentration time curve; BA, bioavailability; CIA, collagen-induced arthritis; C_{max}, drug plasma concentrations at maximum; FITC, fluorescein-5-isothiocyanate; HE, hematoxylin and eosin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 MMP; OA, osteoarthritis; PMN, polymorphonuclear leukocyte; PG, proteoglycan; RA, rheumatoid arthritis; T_{max}, time when plasma concentration reached maximum; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IIC, type II collagen

Introduction

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are involved in connective tissue breakdown both in normal physiological processes, such as development and wound healing (Stamenkovic, 2003), and in several disease states, including atherosclerosis, tumor invasion, ulcerative diseases and arthritic diseases (Fini *et al*., 1996; Curran & Murray, 1999; Herouy *et al*., 2000; Mengshol *et al*., 2002; Jones *et al*., 2003). Rheumatoid arthritis (RA) and osteoarthritis (OA) are chronic arthritic diseases that result in joint destruction and loss of function. This joint destruction is due, in part, to the degradation of extracellular matrix in articular cartilage, mainly composed of fibrillar type II collagen (IIC) and aggregating proteoglycans (PGs). In the pathogenesis of RA and OA, degradation of cartilage collagen matrix is primarily accomplished by MMPs that include collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10) and membrane type MMPs (MT-MMPs). Collagenases can cleave the intact triple helix of collagen, at single site three-quarters of the distance from the amino-terminus, under physiological conditions (Brinckerhoff, 1991; Mengshol *et al*., 2002). Gelatinases are well known for their ability to degrade the basement membrane and partially degraded collagen (gelatin), working in concert with collagenases to digest collagen matrix. Gelatinases are also involved in bone resorption and angiogenesis, which contribute to joint destruction either directly or indirectly (Itoh *et al*., 1998; Sang, 1998; Koivunen *et al*., 1999; Delaisse *et al*., 2000;

*Author for correspondence;
E-mail: takeshi_ishikawa@po.fujisawa.co.jp
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Jackson *et al*., 2001). Membrane type 1 MMP (MT1-MMP)/MMP-14 has been identified as an activator of pro-MMP-2 (Strongin *et al*., 1995). MMP-14 is also called a tethered collagenase since MMP-14 can digest fibrillar collagens like collagenases (Ohuchi *et al*., 1997; Holmbeck *et al*., 2004).

Clinically, current treatments for RA and OA reduce pain and swelling of the joint but generally do little to block the cartilage degradation underlying these symptoms (Cawston & Rowan, 1998). Although recent anticytokine agents and other biological response modifiers retard the progression of joint damage, the long-term clinical effects of these agents remain to be elucidated. Moreover, these agents are limited due to several problems, such as infectious adverse events, rebound of symptoms, short half-lives or high treatment costs (Bemelmans *et al*., 1994; Lipsky & Kavanaugh, 1999; Kalden, 2001; Ellerin *et al*., 2003). Ultimately, this progressive cartilage degradation may leave joint replacement as the only viable therapeutic option. Therefore, there is an urgent need for the development of cartilage protective drugs. Despite the relevance of MMPs to cartilage degradation (Vincenti *et al*., 1994), few MMP inhibitors have entered clinical trials for RA or OA and none have so far been successful. Trocade is the only MMP inhibitor to have completed clinical trials designed to assess its efficacy, but it did not prevent progression of joint damage in patients with RA (Close, 2001; Jackson *et al*., 2001).

FR255031 (2-[(7*S*)-7-[5-(4-ethylphenyl)-2-thienyl]-1,1-dioxido-4-(2-pyridinylcarbonyl)hexahydro-1, 4-thiazepin-7-yl]-*N*-hydroxyacetamide) is a novel synthetic MMP inhibitor, different from Trocade in several characteristics (Figure 1). FR255031 inhibits collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9) and MT-MMP (MMP-14) *in vitro*. In contrast, Trocade is an inhibitor of collagenase and MT-MMP. In the present study, we compared the ability of these two MMP inhibitors to prevent cartilage degradation in rat collagen-induced arthritis (CIA). CIA is an autoimmune arthritis model induced in susceptible strains of mice, rats or monkeys by immunization with IIC (Trentham *et al*., 1977; Stuart *et al*., 1979; Courtenay *et al*., 1980; Trentham, 1982; Hart *et al*., 1998). This animal model has been widely used to investigate pathogenic mechanisms in RA, such as erosion of bone and articular cartilage, pannus formation and infiltration of inflammatory cells, and also to evaluate potential new therapeutic agents. We demonstrated that FR255031 significantly inhibited cartilage degradation in the rat CIA model, although Trocade failed to prevent the degradation. These results suggest that gelatinases, in cooperation with collagenases and MT-MMP, play an important role in cartilage matrix degradation and that inhibition of collagenase, gelatinase and MT-MMP is recommended in the treatment of arthritic joints.

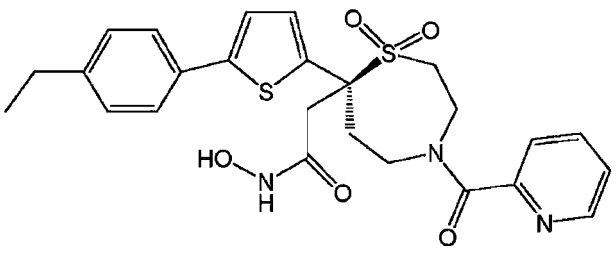


Figure 1 Chemical structure of FR255031, a matrix metalloprotease inhibitor.

Methods

Drugs

FR255031 and Trocade were prepared at Fujisawa Pharmaceutical Co., Ltd (Ibaraki, Japan).

Enzyme inhibition assays

Human MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 were obtained from Yagai (Yamagata, Japan). Culture media of rat synovial fibroblasts with collagenase and gelatinase activities were prepared as reported previously (Hashida *et al*., 1996). Briefly, synovial fibroblasts obtained from rat knee joints of normal female Lewis rats were stimulated with short-term culture media from casein-induced rat peritoneal polymorphonuclear leukocytes (PMNs). Rat synovial fibroblast culture media were then activated by incubation with 1 mM *p*-aminophenylmercuric acetate (Sigma, St. Louis, MO, U.S.A.) overnight at 4°C before rat collagenase and gelatinase assay. MMP-1 activity was estimated at 37°C for 2 h using a Type I collagenase activity assay kit containing fluorescein-5-isothiocyanate (FITC)-labeled type I collagen (Yagai). MMP-8 and rat collagenase activities were estimated at 37°C for 2 h using a Type II collagenase activity assay kit containing FITC-labeled type II collagen (Yagai). MMP-2, MMP-3, MMP-9 and rat gelatinase activities were estimated at 42°C for 2 h using a Type IV collagenase activity assay kit containing FITC-labeled type IV collagen (Yagai). MMP-13 activity was estimated at room temperature (RT) for 30 min using an Arthrogen-CIA MMP-13 kit obtained from Chondrex (Redmond, WA, U.S.A.). MMP-14 activity was estimated at 37°C for 10 min using a MMP-14 colorimetric assay kit (Biomol, Plymouth Meeting, PA, U.S.A.). MMP activities except MMP-14 activity were measured with a spectrofluorophotometer (Spectrafluor plus, Tecan, Maennedorf, Switzerland). The reaction of MMP-14 was measured at 412 nm with a spectrophotometer (SpectraMax 250, Molecular Devices, Sunnyvale, CA, U.S.A.). All reactions for estimation of IC_{50} values for MMP subtypes were performed according to the manufacturer's instructions. Each IC_{50} value was estimated by log conversion following simple linear regression.

A disintegrin and metalloproteinase with thrombospondin repeats-4 (ADAMTS-4/Aggrecanase-1) assay was performed according to the method of Miller *et al*. (2003) with some modifications. Briefly, microtiter plates were coated with streptavidin (Vector Laboratories, Burlingame, CA, U.S.A.). After the streptavidin-coated plate was washed with PBS containing 0.05% Tween-20, a biotinylated substrate peptide QTVTWPDMELPLPRNITEGEARGSVILTVKSVVYGLR ($10\,\mu g\,ml^{-1}$), manufactured at Toray (kanagawa, Japan), was added to each well and incubated for 1 h at 37°C. After blocking with PBS containing 1% BSA, anti-substrate peptide antibody, which specifically recognizes SVVYGLR sequence, prepared in our laboratory, was added to each well and incubated for 1 h at 37°C. After washing with PBS, $10\,\mu g\,ml^{-1}$ of human ADAMTS-4 (Chemicon International, Temecula, CA, U.S.A.) was added to each well and incubated in the presence or absence of inhibitors for 7 h at 37°C. After washing, horseradish peroxidase-conjugated anti-human IgG (Zymed Laboratories, South San Francisco, CA, U.S.A.) was added to each well and incubated for 30 min at RT.

Supersensitive TMB solution (Sigma) was added and incubated for 20 min at RT. The reaction was stopped by the addition of 2 N H_2SO_4 and read at 450 nm. In our assay systems, a biotinylated substrate and anti-substrate antibody are different from those in the assays of Miller *et al*. In our biotinylated substrate peptide, six amino-acid-sequence (SVVYGLR) was added to Miller's substrate at C-terminal end. Our anti-substrate antibody recognizes this six amino-acid sequence, and specifically interacts with undigested substrate, but not with aggrecanase-digested substrate. This aggrecanase assay system had been validated using aggrecanase-specific inhibitor, ST-109 (Pratta *et al*., 2003), before drug evaluations.

Cell-based tumor necrosis factor (TNF) inhibition assays

The cell-based activities of FR255031 and Trocade were evaluated in human THP-1 and rat splenocytes for inhibition of lipopolysaccharide (LPS)-induced TNF-α release. Human THP-1 cells were treated with LPS ($10\,\mu g\,ml^{-1}$) (serotype B5:055, Sigma) for 18 h in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Moregate biothech, Bulimba, Qld, Australia). Rat splenocytes were treated with LPS ($1\,\mu g\,ml^{-1}$) for 18 h in DMEM supplemented with 1% FBS. At the end of incubation period, media were collected and frozen at −80°C. The concentrations of human TNF-α were determined by an ELISA assay using MAb1 as capture antibody, and MAb11 as detection antibody (Becton Dickinson, San Diego, CA, U.S.A.) according to the manufacturer's instructions. The concentrations of rat TNF-α were determined by an ELISA assay using TN 3-19.12 as capture antibody, and polyclonal anti-Ms/Rt as detection antibody (Becton Dickinson) according to the manufacturer's instructions.

Induction of arthritis

Female, 7-week-old Lewis rats were purchased from Charles River Japan Inc. (Kanagawa, Japan) and bred in a clean atmosphere with 12 h light/dark cycles. Rats were fed standard rodent chow *ad libitum* and were free from infectious diseases. They were allowed 1 week to adapt to their environment and used at 8 weeks of age. All experimental procedures were performed according to guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical Co., Ltd. Bovine IIC (Collagen Research Center, Tokyo, Japan) was dissolved in 0.1 M acetic acid ($2\,mg\,ml^{-1}$) overnight at 4°C. The solution was emulsified in an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Rats were randomized and grouped ($n=10$) for drug treatment based on hind paw volume and body weight. Each rat was immunized with 0.5 ml of the cold emulsion by more than 10 intradermal injections on the back and by two injections into the base of the tail on day 0, and 0.1 ml of the cold emulsion by two booster injections into the base of the tail on days 7 and 14.

Drug treatment

Effect of FR255031 or Trocade on rat CIA model was evaluated independently. FR255031 was dissolved in Polyethylene Glycol #400 (PEG400) (Nacalai Tesque, Kyoto, Japan), and Trocade was dissolved in distilled water (DW). Vehicle (PEG400 for FR255031 or DW for Trocade), FR255031 (10, 32 and $100\,mg\,kg^{-1}$) and Trocade (10, 32 and $100\,mg\,kg^{-1}$) were orally administered twice a day from days 1 to 28.

Evaluation of CIA

Body weight for each rat was measured on days 0 and 28. The volume of both hind paws was measured on days 0 and 28 by a water displacement method using a plethysmometer for rats (MK-550, Muromachi Kikai Co., Ltd, Tokyo, Japan). Paw swelling was presented as the mean of increase in the volume of both hind paws.

For measurement of serum anti-IIC antibody, peripheral blood was obtained from the abdominal artery of rats on day 29 after general anesthesia by inhalation of diethyl ether and serum was collected by centrifugation. Anti-IIC antibody in the serum was measured by an enzyme-linked immunosorbent assay. Microtiter plates were coated with native bovine IIC (Collagen Research Center) at $50\,\mu g\,ml^{-1}$ in PBS containing 0.1% BSA and 0.05% Tween-20 (0.1% T-PBS). After the IIC-coated plate was washed with 0.1% T-PBS, serum samples were diluted with 0.5% T-PBS, then incubated for 1 h at RT. After washing, peroxidase-conjugated goat anti-rat IgG (ICN-Cappel, Aurola, OH, U.S.A.) was added to each well and incubated for 30 min at RT. A substrate mixture consisting of *o*-phenylendiamine dihydrochlolide (Sigma) and 0.003% H_2O_2 was dissolved in citrate-phosphate buffer before use. The substrate mixture was added and incubated for 20 min at RT. The reaction was stopped by the addition of 6.6% H_2SO_4 and read at 492 nm. Antibody titers were based on an authentic standard (anti-IIC antibody previously prepared from CIA rats in our laboratory). Results for test drugs was calculated using the following formula: % reduction $=(1-B/A)\times 100$, where $A=$CIA control−normal nontreated (NT), $B=$drug treated−NT.

Radiological evaluation of CIA

Right hind paws were removed for radiological scoring. All radiographs were taken with X-ray film (Kodak Diagnostic Film, Ready-Pack, X-OMAT™, Kodak, Rochester, NY, U.S.A.) using MBR-1505R (Hitachi Medical Corporation, Tokyo, Japan). Settings for radiography were 5 mA, 50 kV and 1 min exposure. Films were placed 60 cm below the X-ray source. Radiological scoring was carried out on the basis of bone destruction (no detectable change, 0; damaged, 1) and joint space narrowing (no detectable change, 0; narrowed, 1). Bone destruction was scored independently for each of the following bones of ankle joints: calcaneus, talus, naviculare, metatarsus and distal tibia. Joint space narrowing was independently scored for each of the following two joints: joint between talus and naviculare, and joint between naviculare and cuneiform. The score for each joint specimen was represented as a total of points for the bone destruction scores and joint space narrowing scores. Radiological scoring was performed by a pathologist trained in rat joint pathology.

Histological evaluation of CIA

For the histological evaluation, right hind paws used for radiological scoring were used. Whole ankle joints were

dissected and fixed for 3 days in 10% neutral buffered formalin. After decalcification in 10% formic acid, specimens were paraffin embedded. Tissue sections (2 μm) were stained with hematoxylin and eosin (HE) or Toluidine Blue. Histological analysis was carried out on the basis of bone destruction, cartilage degradation, synovial proliferation and infiltration of inflammatory cells. Severity of lesions was classified into four grades: 0, no detectable change; 1–3, slight to severe. Histological parameters were scored by a pathologist trained in rat joint pathology.

Tissue sections prepared from the same preparations used for HE or Toluidine Blue staining were used for immunohistochemistry. Sections (2 μm) were treated with 3% H_2O_2 for 30 min at RT. After rinsing, sections were blocked with normal serum for 10 min at RT and incubated overnight at 4°C with primary antibodies specific for IIC (F-57, DFK, Toyama, Japan). Thereafter, sections were incubated with goat anti-mouse IgG conjugated with peroxidase (Jackson Immunoresearch, West Grove, PA, U.S.A.) for 30 min at RT. After development of the peroxidase staining, counterstaining was carried out with Mayer's hematoxylin.

Pharmacokinetic evaluation of FR255031 and Trocade

Sprague–Dawley (SD) rats (8 weeks old) and Lewis rats (8 weeks old), used in these assays, were purchased from Charles River Japan Inc. and were divided into six groups of three rats to be given single doses of FR255031 or Trocade. One group of male SD rat was given a single intravenous (i.v.) bolus administration of FR255031 at 3.2 mg kg^{-1} *via* femoral vein. Blood samples were taken 5, 15 and 30 min and 1, 2, 4, 6 and 8 h after the i.v. bolus dose from the cannula in the femoral artery in each animal. Second group of male SD rat was given a single oral administration of FR255031 at 10 mg kg^{-1}. Blood samples were taken 15 and 30 min and 1, 2, 4, 6 and 8 h after the oral dose from the tail vein in each animal. Third or fourth group of female Lewis rat was given a single oral administration of FR255031 at 32 or 100 mg kg^{-1}. Blood samples were taken 30 min and 1, 2, 4 and 6 h after the oral dose from the orbital vein in each animal under anesthesia by diethyl ether. Fifth or sixth group of female Lewis rat was given a single oral administration of Trocade at 32 or 100 mg kg^{-1}. Blood samples were taken same time course as third or fourth group in each animal. The anticoagulant used was heparin (Shimizu pharmaceutical, Sizuoka, Japan) and plasma was separated by centrifugation and then stored at −20°C for analysis. After adding methanol, the plasma mixture was vortexed and centrifuged at 10,000 × g for 5 min, and an aliquot of the supernatant was injected into HPLC or HPLC with tandem mass spectrometry (HPLC/MS/MS) for the quantitation of the plasma concentration.

Determination of plasma concentration of FR255031 was performed by HPLC analysis. The HPLC system used was an LC module I plus (Millipore Co., Milford, MA, U.S.A.). The column for analysis was an Inertsil ODS-3 (5 μm, 150 × 4.6 mm^2) (GL Science Inc., Tokyo, Japan). The eluent was monitored at a wavelength of UV 298 nm. The column was used at RT. The mobile phase consisted of 0.02 M phosphate buffer (pH 3)/CH_3CN (57/43), with a flow rate of 1.0 ml min^{-1}.

Determination of plasma concentration of Trocade was performed by HPLC/MS/MS analysis. The HPLC system used was an Agilent series 1100 liquid chromatography system (Agilent, Palo Alto, CA, U.S.A.). The column for analysis was an Inertsil ODS-3 (5 μm, 150 × 2.1 mm^2) (GL Science Inc.). The column was used at 40°C. The mobile phase consisted of 0.1% HCOOH/CH_3CN (60/40), with a flow rate of 0.2 ml min^{-1}. Selective detection was accomplished on a Sciex API 2000 (Applied Biosystems, Foster City, CA, U.S.A.) mass spectrometer in the multiple reaction monitoring mode by electrospray ionization.

Pharmacokinetic parameters were calculated from the individual data by use of model-independent methods and quoted as the average data from the three animals in each group. Area under the plasma concentration time curve from 0 to 8 ($AUC_{0-8\,h}$) or from 0 to 6 ($AUC_{0-6\,h}$) was calculated by the linear trapezoidal method. Drug plasma concentrations at maximum (C_{max}) and time when plasma concentration reached maximum (T_{max}) were observed values. Bioavailability (BA) was calculated from the dose-adjusted $AUC_{0-8\,h}$ values of the oral dose and the i.v. dose.

Statistical analysis

Data are expressed as mean ± s.e. Statistical significance of differences was assessed by Dunnett's multiple comparison test following one-way analysis of variance and Student's *t*-test for comparison of two samples. In the case of histological or radiological scoring, Wilcoxon's nonparametric analysis of variance for comparison of two samples was performed. $P<0.05$ was set as the level of significance.

Results

Inhibitory activities of FR255031 and Trocade on various enzymes and TNF-α release in cell-based assays

Inhibitory effects of FR255031 and Trocade on various enzymes were examined (Table 1). FR255031 inhibited human

Table 1 Inhibitory activities of FR255031 and Trocade on various enzymes and TNF-α release in cell-based assays

	IC_{50} (nM)	
	FR255031	*Trocade*
Human		
Collagenase		
MMP-1	77.8	1.77
MMP-8	3.12	2.92
MMP-13	1.13	7.28
Gelatinase		
MMP-2	91.1	3420
MMP-9	3.94	150
Stromelysin		
MMP-3	>10,000	694
MT-MMP		
MMP-14	1.80	7.84
ADAMTS		
Aggrecanase-1	>10,000	>10,000
TNF-α release	>10,000	>10,000
Rat		
Collagenase	75.8	22.2
Gelatinase	82.1	667
TNF-α release	>10,000	>10,000

Table 2 Effect of FR255031 and Trocade on body weight, paw swelling and serum anti-IIC antibody

Treatment	*Dose* (mg kg^{-1})	*Body weight* (g)	*Paw swelling* (ml)	*Serum anti-IIC antibody (% inhibition)*
NT	—	221±1	0.01±0.01	100
CIA control	—	184±3**	0.45±0.04**	0
	10	192±4	0.46±0.06	12.3
FR255031	32	192±4	0.51±0.08	−6.2
	100	199±4$^{\#}$	0.36±0.10	20.7
NT	—	215±2	0.07±0.01	100
CIA control	—	180±3**	0.61±0.02**	0
	10	179±2	0.63±0.04	−1.4
Trocade	32	181±2	0.58±0.04	4.6
	100	181±3	0.66±0.05	6.4

Body weight was measured on day 28.
Paw swelling on day 28 was expressed as a mean of changes in the both hind paws.
Anti-IIC antibody level in serum on day 29 was measured by ELISA and the results of test compounds were expressed as % inhibition.
Values were shown as the mean±s.e.
**$P<0.01$ compared to NT.
$^{\#}P<0.05$ compared to CIA control.

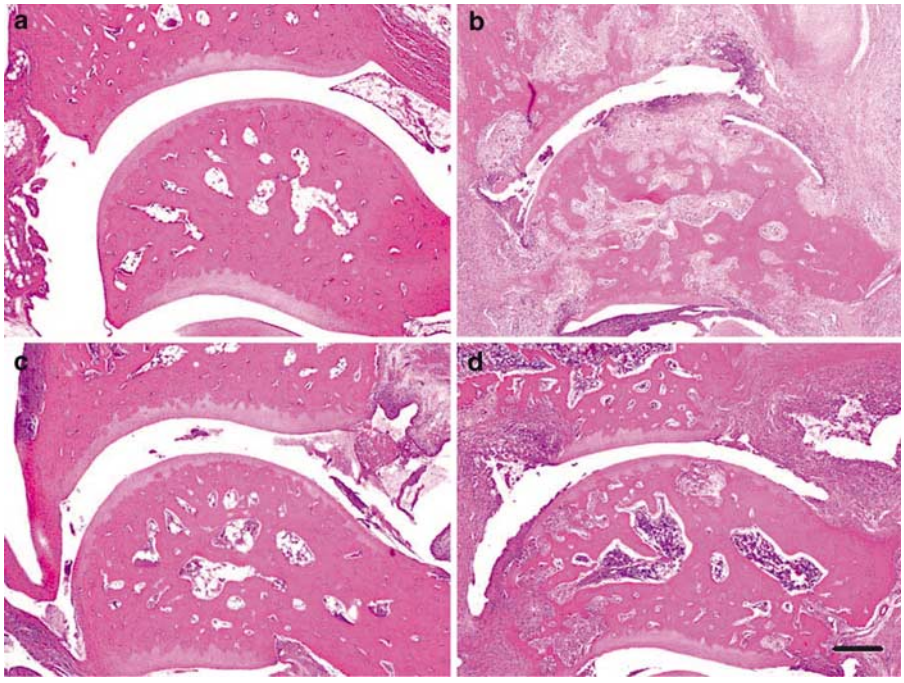


Figure 2 Histological analysis of ankle joints of NT rat (day 29), CIA rat, FR255031- (100 mg kg^{-1}) treated rat with arthritis and Trocade- (100 mg kg^{-1}) treated rat with arthritis. Tissue sections from each group were stained with HE. Sections from an NT rat (a) exhibit normal histological features with intact articular cartilage and no evidence of inflammation or erosion. CIA control rats showed severe joint damage, as evidenced by erosion, pannus formation and infiltration of inflammatory cells (b). FR255031-treated rats with arthritis showed intact articular cartilage and slight bone resorption with mild inflammatory cell infiltration into synovium (c). Trocade-treated rats showed severe joint damage (d) that was similar to CIA rats. Scale shown on the right-hand side of (d) is equivalent to 0.3 mm.

collagenases (MMP-1, MMP-8 and MMP-13), human gelatinases (MMP-2 and MMP-9) and human MMP-14 with IC_{50} values of 77.8, 3.12, 1.13, 91.1, 3.94 and 1.80 nM, respectively. FR255031 did not inhibit human MMP-3 up to 10^{-5} M. FR255031 also inhibited both rat collagenase and rat gelatinase with similar potency (IC_{50} values of 75.8 and 82.1 nM, respectively) under our experimental conditions. Trocade potently inhibited collagenase subtypes and MMP-14, and only weakly inhibited gelatinase subtypes and human MMP-3. Both FR255031 and Trocade did not inhibit aggrecanase-1 up to 10^{-5} M. Moreover, both FR255031 and Trocade also did not affect LPS-induced TNF-α release in human and rat cell-based assays.

Evaluation of CIA

In this CIA model, bodyweight loss and paw swelling manifested from day 7 after first immunization and continued until at least day 28, when the experiment was terminated (data not shown). The level of serum anti-IIC antibody on day 29

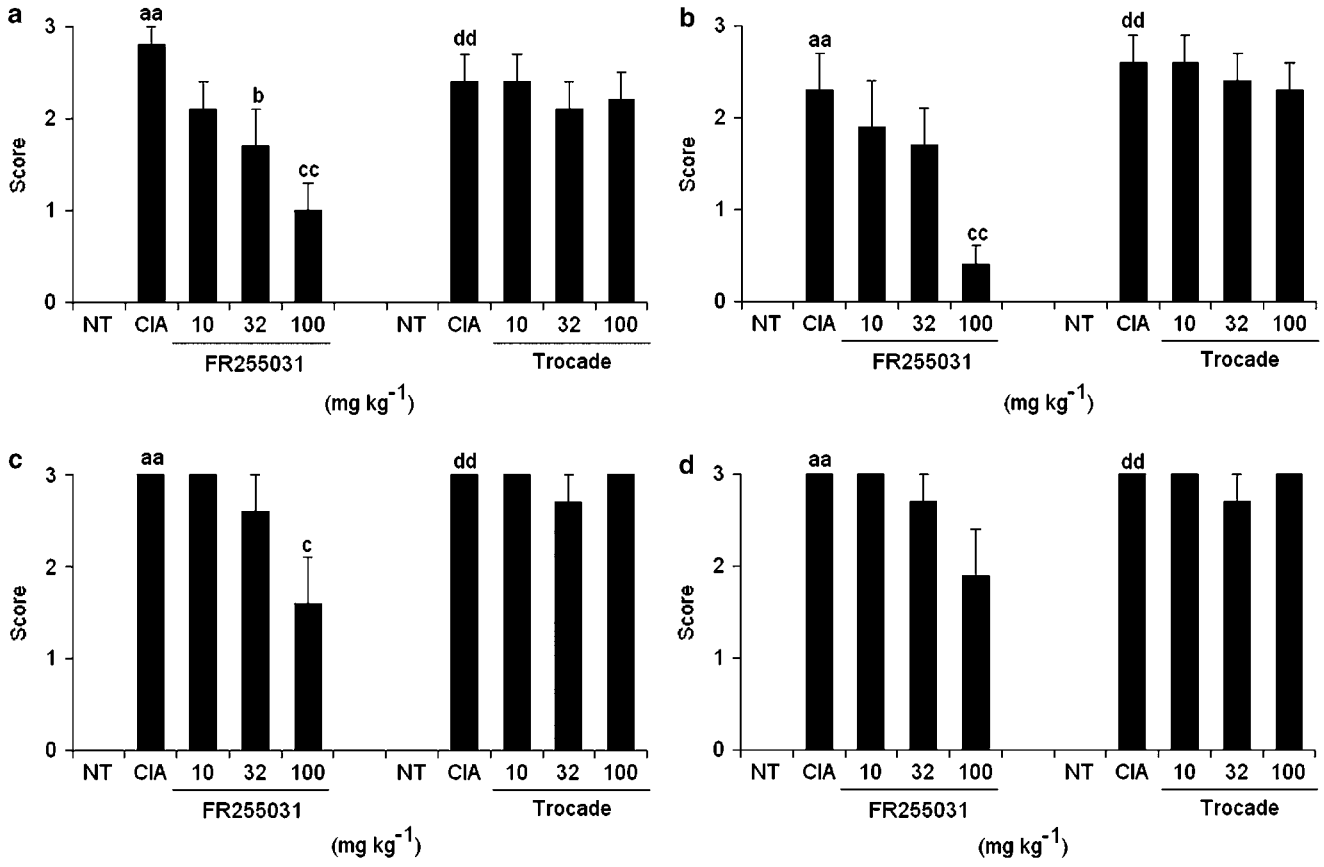


Figure 3 Inhibitory effect of FR255031 and Trocade on histological scores in ankle joints of rats with CIA. Cartilage degradation (a), bone destruction (b), synovial proliferation (c) and infiltration of inflammatory cells (d) were graded as described in Methods. Values are shown as mean ± s.e. For FR255031 study, aa; $P<0.01$ compared with NT. b; $P<0.05$ compared with CIA control. c or cc; $P<0.05$ or 0.01 compared with CIA. For Trocade study, dd; $P<0.01$ compared with NT.

was also significantly elevated. FR255031 (100 mg kg^{-1}) was slightly effective for the recovery of body weight loss on day 28, and also tended to suppress paw swelling. Trocade did not suppress body weight loss or paw swelling. Both FR255031 and Trocade had no effect on serum anti-IIC antibody levels (Table 2).

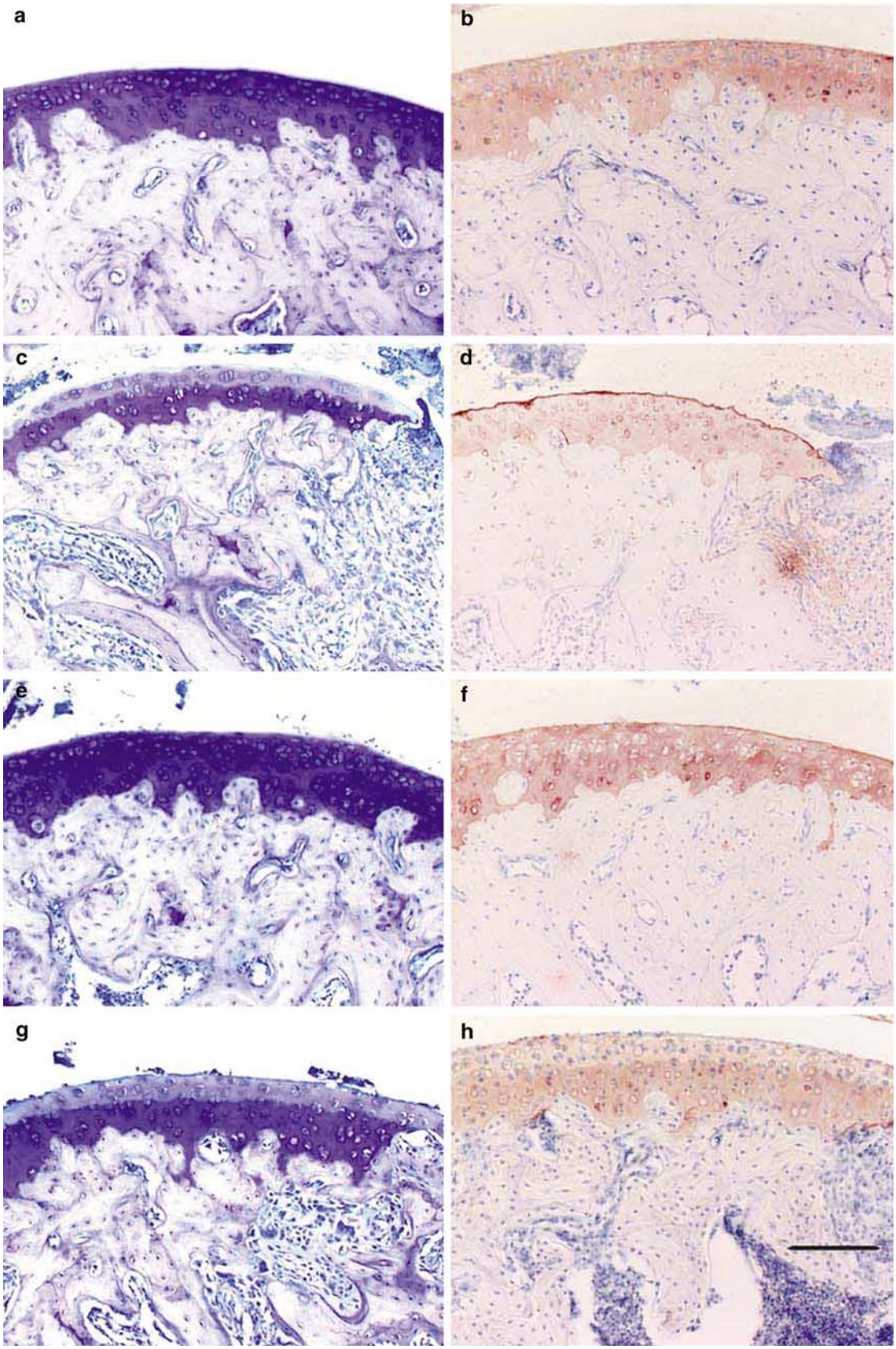
Histological evaluation of CIA

Photomicrographs of sections stained with HE illustrate the disease severity and effect of compound treatment on joint histology (Figure 2). No inflammation or tissue destruction was seen in HE sections from NT rats (Figure 2a). In contrast, ankle joints of CIA control rats showed severe joint destruction with extensive inflammation, and erosion of cartilage and bone. Enlarged cavities as a result of the erosion were filled with synovial fibroblasts and inflammatory cells (Figure 2b). FR255031 at a dose of 100 mg kg^{-1} clearly inhibited the joint destruction, although a slight hypertrophy of synovium and mild inflammatory cell infiltration remained (Figure 2c). Trocade could not prevent those arthritic changes (Figure 2d).

Scoring of histological lesions in the sections from ankle joints stained with HE was performed on the basis of cartilage degradation, bone destruction, synovial proliferation and infiltration of inflammatory cells. Histological analysis revealed FR255031 markedly inhibited cartilage degradation in a dose-dependent manner (Figure 3a). FR255031 also showed statistically significant effects on bone destruction and pannus formation at a dose of 100 mg kg^{-1} (Figure 3b and c). The inhibitory effect of FR255031 on cartilage degradation was more prominent than on other parameters. Trocade did not prevent all four parameters (Figure 3a–d).

A marked loss of Toluidine Blue staining was observed in ankle joints of CIA rats, in contrast to that of NT rats (Figure 4a and c). FR255031 clearly inhibited the destaining, that is,

Figure 4 Histological analysis of ankle joints of NT rat (day 29) (a and b), CIA control rat (c and d), FR255031-(100 mg kg^{-1}) treated rat with arthritis (e and f) and Trocade- (100 mg kg^{-1}) treated rat with arthritis (g and h). Tissue sections from each group were stained with Toluidine Blue (a, c, e and g), or were immunostained with antibody specific for IIC (b, d, f and h). The normal articular cartilage stained intensely with Toluidine Blue (a), and also immunostained intensely with anti-IIC antibody (b). There was a loss of Toluidine Blue staining in the hyaline cartilage, indicating that PG was depleted from CIA control rat (c). A loss of IIC immunostaining in the hyaline cartilage was observed, indicating that IIC was released from CIA control rat (d). FR255031-treated rat with arthritis showed intact Toluidine Blue staining or IIC immunostaining of articular cartilage (e and f). Trocade-treated rat showed severe joint damage with loss of PG or IIC in articular cartilage (g and h) that was similar to CIA control rats. Scale shown on the right-hand side of (h) is equivalent to 150 μm.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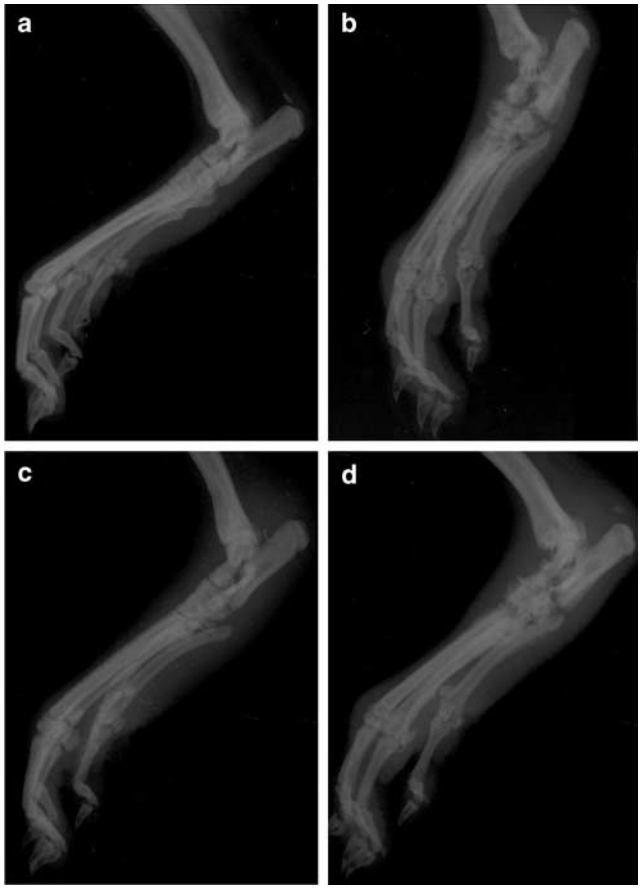

Figure 5 Radiographic analysis of ankle joints of NT rat (a), CIA rat (b), FR255031- (100 mg kg^{-1}) treated rat with arthritis (c) and Trocade- (100 mg kg^{-1}) treated rat with arthritis (d) (day 29). Radiograph shows no joint damage in the right hind paw of NT rat (a). Severe bone erosion and joint space narrowing were observed in CIA rat (b). Those arthritic changes were absent in FR255031- (100 mg kg^{-1}) treated rat with arthritis (c). Trocade (100 mg kg^{-1}) treatment failed to prevent arthritic changes (d).

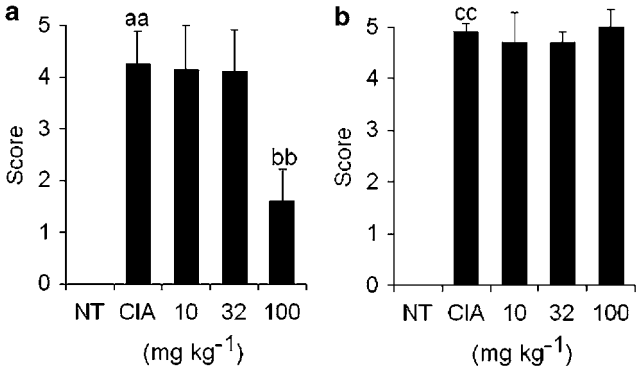

Figure 6 Inhibitory effect of FR255031 (a) and Trocade (b) on radiographic scores in ankle joints of rats with CIA. Radiographic score was determined on the basis of bone destruction and joint space narrowing, as described in Methods. Values are shown as mean±s.e. aa; $P<0.01$ compared with NT. bb; $P<0.01$ compared with CIA. cc; $P<0.01$ compared with NT.

Table 3 Single dose pharmacokinetic parameters of FR255031 and Trocade in the rat

	FR255031	*Trocade*
SD rat		
3.2 mg kg^{-1} i.v. bolus		
$AUC_{(0-8h)}$ (μg h ml^{-1})	5.41±1.23	
$t_{1/2\beta}$ (h)	0.37±0.07	
V_{ss} (l kg^{-1})	0.14±0.02	
$Cltot_{0-8h}$ (ml min^{-1} kg^{-1})	11.34±3.26	
10 mg kg^{-1} p.o.		
C_{max} (μg ml^{-1})	0.615±0.014	
T_{max} (h)	0.42±0.08	
$AUC_{(0-8h)}$ (μg h ml^{-1})	1.69±0.07	
BA (%)	9.99±0.43	
Lewis rat		
32 mg kg^{-1} p.o.		
C_{max} (μg ml^{-1})	4.451±0.580	1.522±0.291
T_{max} (h)	0.50±0.00	0.50±0.00
$AUC_{(0-6h)}$ (μg h ml^{-1})	5.511±0.774	1.458±0.152
100 mg kg^{-1} p.o.		
C_{max} (μg ml^{-1})	20.202±4.208	13.302±3.662
T_{max} (h)	0.50±0.00	0.50±0.00
$AUC_{(0-6h)}$ (μg h ml^{-1})	26.467±2.840	12.077±2.899

Pharmacokinetic parameters were: area under the plasma concentration time curve from 0 to 8 h or from 0 to 6 h ($AUC_{(0-8h)}$ or $AUC_{(0-6h)}$); half-lives ($t_{1/2\beta}$) for second phase; volume of distribution of a steady state (V_{ss}); total clearance from 0 to 8 h ($Cltot_{0-8h}$); maximal concentration observed in plasma (C_{max}); time when concentration of FR255031 or Trocade in plasma was observed to be at a maximum (T_{max}) and bioavailability (BA). Values were shown as the mean±s.e.

inhibition by FR255031 resulted in retention of PG in articular cartilage (Figure 4e). The superficial area of articular cartilage in rats treated with Trocade had PG depletion, as indicated by lack of staining with Toluidine Blue (Figure 4g). Immunostaining with anti-IIC antibody revealed collagen matrix degeneration, as indicated by a marked loss of staining that occurred in CIA control rats (Figure 4d). These results were similar to those obtained from Toluidine Blue staining. FR255031 markedly inhibited destaining, but Trocade did not block the loss of anti-IIC staining in the superficial area of articular cartilage (Figure 4f and h).

Radiological evaluation of CIA

Radiographic severity of joint destruction is shown in Figure 5. In the ankle joint of CIA control rats, bone erosion and joint space narrowing was detected (Figure 5b). Arthritic changes in FR255031- (100 mg kg^{-1}) treated rats were significantly reduced when compared with those in CIA control rats (Figure 6a). These results were similar to those obtained from histological examination. Trocade did not prevent the joint destruction, even as assessed by radiological evaluation (Figure 6b).

Pharmacokinetic evaluation of FR255031 and Trocade

Pharmacokinetic parameters of FR255031 and Trocade are presented in Table 3. After an i.v. bolus dose of 3.2 mg kg^{-1} in the SD rat, FR255031 was distributed with a V_{ss} of 0.141 kg^{-1} and then cleared with a $t_{1/2}$ of 0.37 h. Clearance was

11.34 ml min^{-1} kg^{-1}. Following oral administration at 10 mg kg^{-1} in the SD rat, FR255031 in the blood increased rapidly (T_{max}, 0.42 h) and its BA at this dose was approximately 10%. Plasma concentration of FR255031, after oral administration at 32 or 100 mg kg^{-1} in the Lewis rat, which was also used in CIA model, was relatively higher than that of Trocade. Nevertheless, the level of Trocade at 100 mg kg^{-1} was much higher than that of FR255031 at 32 mg kg^{-1}.

Discussion

In the present study, we estimated the inhibitory activity of two MMP inhibitor compounds with different characteristics, FR255031 and Trocade, in a rat CIA model to determine the role of those target MMP subtypes in joint destruction. The present study focused on a rat CIA model because of the many features this model has in common with human RA (Joe & Wilder, 1999). In particular, accumulation of IIC- or aggrecan-breakdown products generated by MMPs or other enzymes are observed both in CIA animals (Singer *et al*., 1995; 1997; Mudgett *et al*., 1998; van Meurs *et al*., 1999; Lubberts *et al*., 2000; Price *et al*., 2002), and in RA and OA patients (Dodge & Poole, 1989; Fosang *et al*., 1996; Lark *et al*., 1997). Despite widespread use of the CIA model and the relevance between MMP subtypes and joint destruction, surprisingly few studies exist evaluating the effect of MMP inhibition using the CIA model. As far as we know, only one published study exists, describing that disruption of the stromelysin 1 gene neither prevents nor reduces cartilage destruction associated with CIA (Mudgett *et al*., 1998). For this reason, we first estimated the ability of synthetic MMP inhibitors in preventing cartilage degradation in the rat CIA model.

FR255031 ameliorated disease activities, as indicated by chronic inflammation and joint damage in rat CIA; however, Trocade did not prevent those disease activities, although plasma concentration profile of Trocade was comparable to that of FR255031. FR255031 is an inhibitor of collagenase, gelatinase and MT-MMP, whereas Trocade is a collagenase and MT-MMP inhibitor. These results suggest that, as well as collagenase and MT-MMP, gelatinase is involved in the pathogenesis of arthritis in this model.

FR255031 suppressed body weight loss, paw swelling and pannus formation in CIA. None of these effects were found in Trocade-treated rats. This suggests that inhibition of gelatinase activity might act on chronic inflammation in the pathogenesis of arthritis. BAY 12-9566 was originally developed as an inhibitor of MMP-2, MMP-3 and MMP-9, with a similar effect on chronic inflammation, as shown by synovial necrosis and capsular fibrosis, and paw swelling in the rat adjuvant arthritis model (Hamada *et al*., 2000).

Histological and radiological evaluation revealed that oral administration of FR255031 clearly inhibited joint destruction, especially cartilage degradation, in a dose-dependent manner. The inhibitory effect of FR255031 on cartilage degradation was more potent than that on bone destruction and pannus formation. These results suggest that FR255031, by inhibiting MMP activities, can directly protect articular cartilage. Articular cartilage from Trocade-treated rats had IIC degeneration, as indicated by a loss of anti-IIC immunostaining. Although each collagenase subtype or MMP-14 by itself can cleave soluble IIC *in vitro*, it might be insufficient to cause degeneration of fibrillar IIC embedded in articular cartilage *in vivo*. In contrast, FR255031 suppressed both IIC degeneration and PG depletion. Moreover, FR255031 inhibited structural damage in articular cartilage. The protection of structural damage might be due to the retention of fibrillar IIC and aggregating PG, two major components of articular cartilage thought to be responsible for tensile strength and compressive stiffness, respectively, necessary for normal articulation and function (Poole *et al*., 2001). Articular cartilage preserving these two components may be able to resist factors like mechanical loading, which cause cartilage deformation and degeneration.

Why FR255031, but not Trocade, can inhibit PG depletion remains to be elucidated, but at least three explanations could be mentioned as follows. First, we cannot exclude the possibility that FR255031 might inhibit another enzyme not examined in this study, such as aggrecanase-2, that leads to PG depletion. Second, FR255031 may inhibit PG degeneration by blocking aggrecanase expression. Gelatinase subtypes, that is, MMP-2 and MMP-9 could induce proteolytic activation of latent transforming growth factor-β (TGF-β). TGF-β is reported to regulate aggrecanase-1 expression in fibroblast-like synoviocyte (Yu & Stamenkovic, 2000; Yamanishi *et al*., 2002). It is possible that FR255031 may act on aggrecanase expression *via* blocking TGF-β activation. FR255031 also suppressed expansion of synovial tissue, which is one of major source of aggrecanase, in the arthritic joint of CIA rat. At last, FR255031 may inhibit PG degeneration by blocking IIC cleavage. Our results favor the concept that degradation of the collagen fibrillar network may lead to a loss of PG. Since the PG aggrecan is arranged in molecular aggregates with hyaluronic acid, which interacts with IIC (Poole *et al*., 1982), the degradation of IIC may thus be indirectly responsible, at least in part, for the local loss of PGs.

Trocade has been tested for its antiarthritic activity in various animal models. Trocade at doses of 2.5–50 mg kg^{-1} reduce arthritic change, as indicated by cartilage degradation or joint damage in these models and its BA (25 mg kg^{-1}) in the rat is 26% (Lewis *et al*., 1997; Brewster *et al*., 1998). However, in clinical trials for RA patients, Trocade treatment was stopped due to the apparent low efficacy of the drug (Close, 2001; Jackson *et al*., 2001). The effect of Trocade in the CIA model used in the current study agrees with the results of clinical trials. In this context, the CIA model might be a suitable model for estimating the clinical effect of candidate drugs with cartilage protective effects. FR255031 is able to prevent progressive joint destruction in CIA, and therefore may be a promising antirheumatic drug.

In conclusion, FR255031, a potent inhibitor of collagenase, gelatinase and MMP-14, prevented progressive joint destruction, especially cartilage degradation in a rat CIA model. These findings suggest that gelatinases are involved in cartilage matrix degradation in concert with collagenases and MT-MMP, and that inhibition of collagenase, gelatinase and MT-MMP is adequate for cartilage protection of arthritic joints.

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