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Inflammation Research

Anti-arthritic effects of KF20444, a new immunosuppressive compound inhibiting dihydroorotate dehydrogenase, on rat collagen-induced arthritis

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Abstract. *Objective and Design:* A newly synthesized inhibitor of pyrimidine *de novo* biosynthesis, KF20444 (6,7-dihydro-10-fluoro-3-(2-fluorophenyl)-5H-benzo [6,7] cyclohepta [1,2-b] quinoline-8-carboxylic acid), was evaluated as an inhibitor of dihydroorotate dehydrogenase (DHO-DHase) and tested in the rat collagen-induced arthritis (CIA) model.
Material and Methods: Female Sprague Dawley rats, 5 weeks-old, were used for evaluation of KF20444 in the CIA model. Arthritis was evaluated by arthritis score, serum anti-type II collagen antibody titer, body weight loss, radiographical and histological changes.
Treatment: KF20444 was orally administered 5 times per week (0.3, 1, 3 mg/kg/day).
Results: KF20444 inhibited rat liver dihydroorotate dehydrogenase *in vitro* with $K_i = 8.5 \pm 3.2$ nM, which was a comparable effect to that of brequinar sodium ($K_i = 25.3 \pm 5.3$ nM). The anti-proliferative effect of KF20444 was caused by cell cycle arrest at the S-phase. Treatment with 3 mg/kg/day of KF20444 completely prevented the development of CIA based on reduction of the arthritis score. The 50% effective dose (ED_{50}) of KF20444 on arthritis score was 0.64 mg/kg. KF20444 ameliorated body weight loss associated with disease onset. The compound also inhibited the increase in serum anti-type II collagen antibody level, and reduced both pannus formation and bone erosion. Importantly, KF20444 suppressed the development of arthritis, even when it was administered after booster immunization of collagen.
Conclusions: KF20444 is a novel immunosuppressant which inhibits DHO-DHase and its effects in CIA suggest that it could be useful in the treatment of rheumatoid arthritis.

Key words: Rheumatoid arthritis – Collagen-induced arthritis – Dihydroorotate dehydrogenase – KF20444 – Cell cycle

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Introduction

KF20444 (6,7-dihydro-10-fluoro-3-(2-fluorophenyl)-5H-benzo [6,7] cyclohepta [1,2-b] quinoline-8-carboxylic acid) was synthesized as one of a variety of derivatives of brequinar sodium (NSC368390; DuP 785). KF20444 exhibited an anti-proliferative activity against both T cells (phytohemagglutinin stimulation, $IC_{50} = 92$ nM) and B cells (LPS stimulation, $IC_{50} = 710$ nM) in mouse [1]. KF20444 also inhibited anti-sheep red blood cells (SRBC) antibody production in mice (ED_{50}: 1.1 mg/kg p.o.) and delayed type hypersensitivity reaction in both mice (ED_{50}: 9.2 mg/kg p.o.) and rats (ED_{50}: 0.6 mg/kg p.o.) [1]. Recently, it was demonstrated that KF20444 prolonged survival of cardiac allografts in rats [2, 3].

Brequinar sodium was developed as an anti-proliferative agent for the treatment of cancer and subsequently as an immunosuppressive agent for the prevention of allograft and xenograft rejection [4–7]. Brequinar sodium inhibits the enzymatic activity of dihydroorotate dehydrogenase (DHO-DHase, [EC 1.3.99.11]), the fourth enzyme of *de novo* pyrimidine synthesis [8]. Inhibition of this enzyme prevents the production of pyrimidine nucleotides necessary for the synthesis of RNA and DNA, thus resulting in reduced cell proliferation [5]. *In vivo*, brequinar sodium suppresses the growth of tumors, the induction of contact sensitivity, the generation of cytotoxic T cells, and the production of antibody [5]. It has become widely accepted that immunosuppression by brequinar sodium is due to the *in vivo* inhibition of pyrimidine synthesis [9]. Recently, brequinar sodium was reported to inhibit tyrosine phosphorylation, and it directly inhibited purified lymphocyte kinase (Lck) and Fyn tyrosine kinase [10]. Another DHO-DHase inhibitor, leflunomide (Arava®), also shows inhibitory activity of cell proliferation and antibody production concomitant with the reduction of pyrimidine nucleotide [11]. This anti-proliferative potency of leflunomide is caused by a cell cycle arrest [12]. Leflunomide has both prophylactic and therapeutic effects in animal models for rheumatoid arthritis (RA) including adjuvant-

induced arthritis [13] and antigen-induced arthritis [14]. The efficacy and safety of leflunomide in a double-blind, randomized trial in RA has been proved [15]. Treatment with leflunomide daily was more effective than placebo and showed similar efficacy to sulphasalazine. These data suggest that inhibition of DHO-DHase may be an attractive target to obtain novel drugs for disease modifying antirheumatic drugs (DMARD). Moreover, the therapeutic potential of treating RA patients with a combination of methotrexate and leflunomide has been reported [16].

Rats develop a polyarthritis upon immunization with type II collagen. Histological and pathological changes in the arthritic joints have a similarity to those of RA, characterized by a chronic inflammatory reaction in synovial tissue, producing pain, disability and eventual destruction of joints [17, 18]. Thus, type II collagen-induced arthritis (CIA) is considered as an experimental model for human RA, and is utilized for development as anti-inflammatory and/or anti-arthritis drugs. In the present study, we clarified the inhibitory activity of KF20444 against DHO-DHase and examined both the prophylactic and sub-therapeutic effects of KF20444 in rat CIA.

Materials and methods

Animals

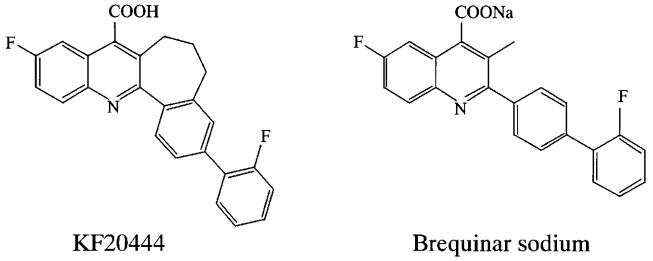
Female, SD 5 week-old rats were purchased from Charles River Japan (Yokohama, Japan). The animals were housed in a room maintained at a temperature of 23 ± 1 °C and a humidity of 55 ± 5%. Food and water were provided *ad libitum*. The animals were allowed 1 week to adapt to their environment and used at 6 weeks of age.

Reagents

KF20444, the free form of brequinar sodium (brequinar; see Fig. 1) and the active metabolite of leflunomide, A77 1726 were all synthesized in our institute. Methotrexate (MTX) was purchased from Lederle Japan, Ltd. (Tokyo, Japan). Prednisolone and naproxen were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Staurosporine was purified in the Technical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.

Collagen immunization

Bovine type II collagen solution, which was purchased from Cosmo Bio Co., Ltd., (Tokyo, Japan), was diluted (3 mg/ml to 1.6 mg/ml) with 10 mM acetic acid and then emulsified in an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI USA). The emulsion (500 µl containing 400 µg collagen) was injected intradermally on the back of rats. Seven days later, 100 µL of the same emulsion was injected intradermally at the base of the tail.



KF20444 Brequinar sodium

Fig. 1. Chemical structures of KF20444 and brequinar sodium.

Drug treatment

Drugs were suspended in 5% arabic gum (Nakarai Tesque Inc., Kyoto, Japan) and administered orally 5 times per week in a volume of 1 ml per 100 g body weight starting on the day of the primary immunization with type II collagen (i.e. from day 0) to the day before the latest day. Immunization with collagen was carried out on Monday in each experiment, and drug was administered from Monday to Friday throughout the experiment. Each experiment finished on day 18 or day 23. Drugs administered orally to rats were as follows: KF20444 (0.3, 1 or 3 mg/kg), MTX (0.1 mg/kg), naproxen (3 mg/kg), prednisolone (3 mg/kg) and brequinar (0.3, 1 or 3 mg/kg). The administered dose of MTX (0.1 mg/kg) was the maximum dose that did not induce myelosuppression [19]. Arabic gum (5 %) was administered as the vehicle control. In the sub-therapeutic experiment, compounds were administered orally 5 times per week from day 7 to the day before the latest day of each experiment.

Arthritis evaluation

Rats were examined for visual appearance of arthritis and the lesion of the each hind paw was graded from 0 to 4 according to the increasing extent of erythema and edema of the periarticular tissue, as described by Trentham et al. [17]. The maximum score was 8 per each animal.

Measurement of serum anti-type II collagen antibody

Anti-type II collagen antibody in the serum was measured by an enzyme linked immunosorbent assay (ELISA) according to a previous report [20]. Affinity purified peroxidase labeled goat anti-rat immunoglobulin (IgG + IgM + IgA; Cappel, Turnhout, Belgium) was used for the detection of rat anti-type II collagen antibodies in the present study. Results are expressed as the absorbance at 490 nm.

Radiographical evaluation

All radiographs were taken with industrial X-ray film IX (Fuji photo film Co. Ltd., Tokyo, Japan) using a soft X-ray machine type SRO-M50 (Sofron Co. Ltd., Tokyo, Japan). The setting for the radiographs were 4 mA, 29 kV and 2 min exposure. The films were placed 50 centimeters below the X-ray source and both right and left hind paws were placed above the films. The radiographs of hind paws were taken at the termination of each experiment. The degree of bone destruction was scored as follows: Each side of each bone in the tarsus was regarded as one region. Cortical destruction was scored as 1. If normal, it was scored as 0. When regions of 2 bones were adjoining, only 1 region was scored. The evaluation allowed for the assessment of 19 distinct regions on each paw. The mean score of both paws gave the bone destruction score. The maximum score was therefore 19.

Histological analysis

For the histological evaluation, ankle joints were immersed in 10% neutral buffered formalin. These joints were decalcified in 10% ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin.

DHO-DHase activity measurement

A partially purified preparation of mitochondria was produced from rat liver (7 week male SD rat, Japan SLC, Shizuoka, Japan) [21]. Measurement of DHO-DHase activity was performed according to Peters et al.

[21] with slight modification. The kinetic parameters (apparent K_m, V_{max}, apparent K_i and K_i') of mitochondrial DHO-DHase were measured using L-dihydroorotate (L-DHO; 50 µM) as a substrate. The rate of conversion of L-DHO to L-orotate was measured by HPLC equipped with SLC-10A system controller, two LC-10AD pumps, SIL-10A autosampler, and SPD-10A UV detector (Shimadzu, Kyoto, Japan). The separation was achieved by isocratic elution with 10 mM potassium phosphate buffer pH 2.7 on a Partisil-10 SAX column (4.6 × 250 mm, Whatman Japan, Tokyo, Japan). The flow rate was 1.5 ml /min, and UV absorbance of the effluent was monitored at 280 nm.

Lck assay

Lck (lymphocyte kinase) was partially purified from bovine thymus tissue (Japan biological material center, Tokyo, Japan) by sequential columns chromatography of DEAE-cellulose, heparin-agarose and butyl-agarose, according to the method of Cushman et al. [22]. The activity of Lck was assayed using [γ-^{32}P] ATP (~6000 Ci/mmol: Amersham Pharmacia Biotec, Tokyo, Japan) and peptide substrate (Tyr-Ala-Glu)$_7$ [23] in the presence of various concentrations of the test compounds. The peptide substrate (Tyr-Ala-Glu)$_7$ was synthesized in our institute. The reaction mixture consisted of peptide substrate (0.5 mg/ml), 12 mM MOPS (pH 7.5), 12 mM $MgCl_2$, 60 µM Na_3VO_4, partially purified Lck (156 µg/ml), 50 µM [γ-^{32}P] ATP and 1% dimethylsulfoxiside, as a carrier for the compounds, in a final volume of 25 µl; incubation was continued for 10 min at 30 °C. The reaction was started by the addition of [γ-^{32}P] ATP at 30 °C and stopped by the addition of 25 µl 10% trichloroacetic acid after 15 min. The acid-precipitable materials were collected on a nitrocellulose membrane filter and washed with 10% trichloroacetic acid. The radioactivity on the filter was measured in Ultima Gold™ scintillation fluid (Packard instrument company, Meroden, CT USA) using a scintillation counter Beckman 6500 (Beckman Coulter Inc., Fullerton, CA USA).

Proliferation assay and cell cycle analyses

Jurkat cells, a human T-cell line from an acute leukemia, were obtained from ATCC (TIB 152) and cultured in RPMI 1640 medium (Sigma-Aldrich Japan, Tokyo, Japan) with 10% fetal bovine serum (Intergen Company, Purchase, NY USA). Proliferation was assessed by the amount of [^{3}H] thymidine (25 Ci/mmol: Amersham Pharmacia Biotec, Tokyo, Japan) incorporation into cells. Jurkat cells (5×10^4 cells/200 µl) were cultured for in the presence or absence of KF20444. After 64 h, [^{3}H] thymidine was added to the cells and the cells were incubated for subsequent 8 h. Cell cycle analyses were carried out using Cycle TEST™ PLUS DNA Reagent Kit from Becton Dickinson Immunocytometry Systems (San Jose, CA USA). Also, cells were analyzed by flow cytometry on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA USA) 6 h after culturing (7.5×10^5 Jurkat cells/1.5 ml) in the presence or absence of A77 1726 or KF20444.

Statistical analyses

Statistical analyses were performed by 1-way ANOVA followed by Dunnett test or Kruskal-Wallis followed by Steel test using the SAS systems for Windows NT version 6.12. P values less than 0.05 were considered to be statistically significant.

Results

Effects of KF20444 on rat CIA model

Treatment with KF20444 (1 mg/kg) almost completely prevented the development of CIA (Fig. 2 A, Table 1). MTX (0.1 mg/kg) partially prevented the development of CIA.

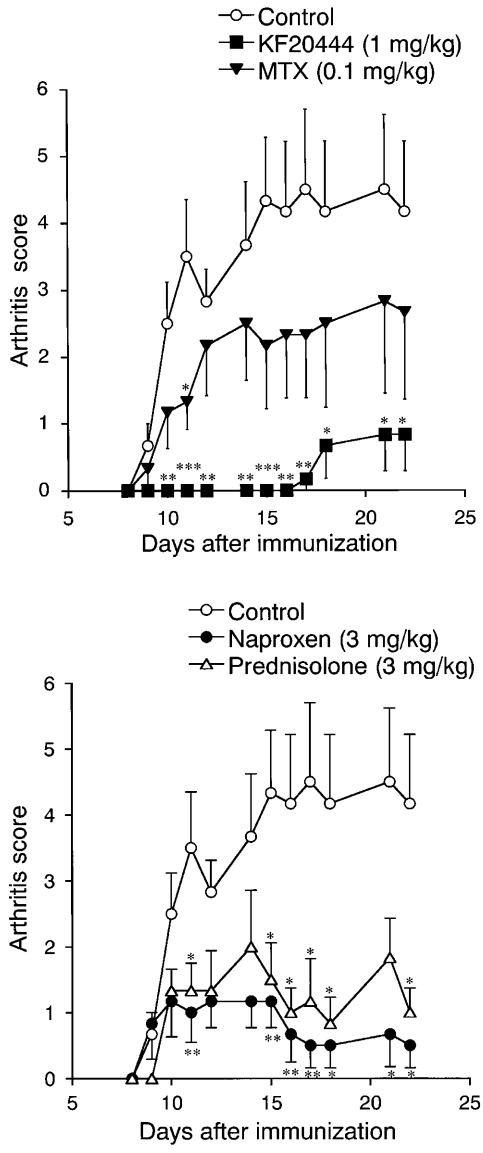


Fig. 2. Effects of KF20444 and various drugs on rat CIA. Rats were immunized with type II collagen by intradermal injection on day 0 and boosted on day 7 as described in methods. Each drug was administered orally 5 times per week from day 0 to day 22. The arthritis score of both hind paws were examined. Effects of KF20444 (1 mg/kg), MTX (0.1 mg/kg) or control on arthritis score are shown in (A). Effects of naproxen (3 mg/kg), prednisolone (3 mg/kg) or control on arthritis score are shown in (B). Results are shown as the mean ± SE (N = 6). *; P < 0.05, **; P < 0.01, ***; P < 0.001 compared with control group.

Both naproxen (3 mg/kg) and prednisolone (3 mg/kg) significantly suppressed arthritis score (Fig. 2B). Although the body weight of vehicle treated rats (control) decreased with the development of arthritis, the body weight of rats treated with KF20444 was maintained as same level as normal rats (Table 2). Extensive erosion of the small bones of the tarsal-metatarsal joint and severe osteoporosis was observed in radiographs of control group at day 23 (data not shown). KF20444, MTX, naproxen and prednisolone, suppressed the destruction of bone (Table 2). The statistics of KF20444 versus MTX, naproxen, and prednisolone gave P values, 0.04, 0.54, and 0.15, respectively.

Table 1. Effects of KF20444 and various drugs on incidence in rat CIA.

Treatment	Dose (mg/kg)	Days after immunization					
		8	9	12	15	18	22
Control	–	0/6[a]	3/6	6/6	6/6	6/6	6/6
KF20444	1	0/6	0/6	0/6	0/6	2/6	2/6
MTX	0.1	0/6	1/6	4/6	4/6	4/6	4/6
Naproxen	3	0/6	2/6	4/6	4/6	2/6	2/6
Prednisolone	3	0/6	0/6	4/6	4/6	3/6	4/6

[a] The incidence of arthritis was examined on the days indicated.

The most remarkable effect of KF20444 was the reduction of anti-type II collagen antibody level in serum (Fig. 3). The level of anti-type II collagen antibody in serum was elevated in all animals of the control group but 86% suppression was observed at day 23 in rats treated with KF20444 ($P = 0.013$). Neither naproxen nor prednisolone suppressed the level of antibody. Histological sections of tarsus joints at 23 days showed an extensive proliferation of the synovium and erosion of both articular cartilage and bone in all animals of the control group (Fig. 4A). A large number of inflammatory cells (neutrophils and mononuclear cells) also migrated into the joint space in the control group. On the other hand, neither synovitis nor erosion of cartilage and bone were observed in joints from rats treated with KF20444 (Fig. 4B). Some infiltration of inflammatory cells was observed in two rats treated with KF20444 that developed slight arthritis from day 18 (data not illustrated).

Dose response analyses for KF20444 and brequinar

KF20444 suppressed the arthritis score in a dose dependent manner (Fig. 5A). Complete suppression was observed at a dose of 3 mg/kg of KF20444 whilst delay and suppression were observed at 1 mg/kg of KF20444. Brequinar also prevented the development of arthritis at 3 mg/kg, but was ineffective at 1 mg/kg (Fig. 5B). KF20444 at both 1 and 3 mg/kg suppressed anti-type II collagen antibody level in serum and bone destruction (Table 3). Brequinar suppressed anti-type II collagen antibody level in serum and bone destruction only at 3 mg/kg (Table 3).

Table 2. Effects of KF20444 and various drugs on body weight change and bone destruction in rat CIA[a].

Treatment	Dose (mg/kg)	Body weight change (g)[b]	Bone destruction score[c]
Normal	–	36.6 ± 5.6**	–
Control	–	13.6 ± 5.6	13.8 ± 0.4
KF20444	1	38.3 ± 2.8**	0.2 ± 0.2*
MTX	0.1	21.7 ± 4.9	7.1 ± 1.8*[+]
Naproxen	3	35.0 ± 3.8*	3.4 ± 2.2*
Prednisolone	3	23.7 ± 4.2	4.6 ± 2.2*

[a] Results are shown as the mean ± SE (N = 6).
[b] Body weight change from day 7 to day 18 was measured.
[c] Radiographs were taken on day 23.
*; $P < 0.05$, **; $P < 0.01$ compared with control group, [+]; $P < 0.05$ compared with KF20444 treated group.

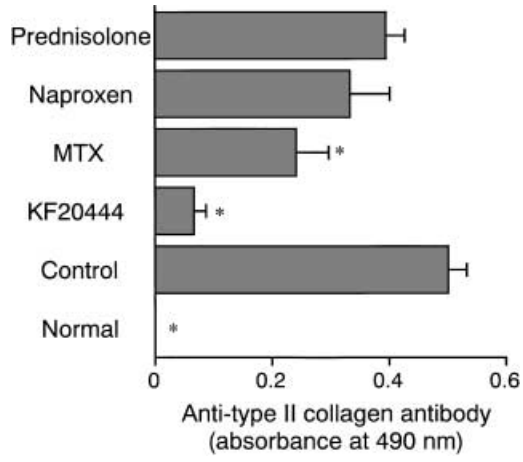


Fig. 3. Effects of KF20444 and various drugs on serum anti-type II collagen antibody level in rat CIA. Anti-type II collagen antibody level in serum on day 23 was measured by ELISA and expressed as the absorbance at 490 nm. The drugs tested with doses in parentheses were: KF20444 (1 mg/kg), naproxen (3 mg/kg), MTX (0.1 mg/kg), prednisolone (3 mg/kg). Results are shown as the mean ± SE (N = 6). *; $P < 0.05$ compared with control group.

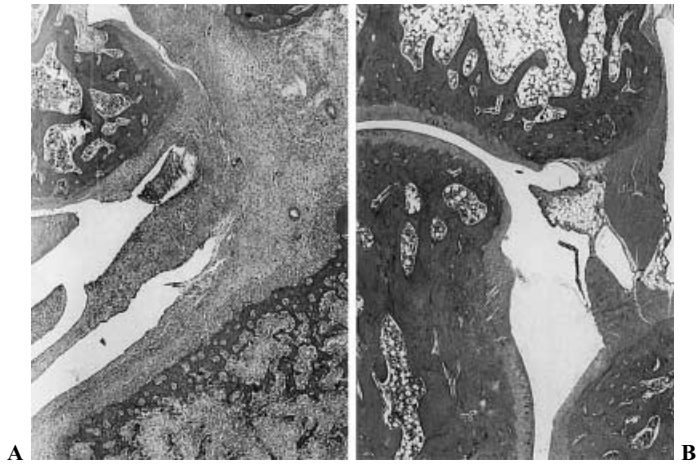


Fig. 4. Hematoxyline-eosin stained paraffin sections of tarsus joints. A control rat immunized with type II collagen and administered with 5% arabic gum showed synovial hyperplasis and erosion of articular cartilage and bone at day 23 (**A**). A rat immunized with type II collagen and administered with KF20444 (1 mg/kg) showed no synovitis and erosion of cartilage at day 23 (**B**). (Original magnification: X 40.)

Sub-therapeutic effects of KF20444 and brequinar on rat CIA

The anti-arthritic activity of KF20444 was assessed when it was administered from day 7, which was the day of second immunization of collagen and just before the development of arthritis. KF20444 (1 mg/kg) suppressed the arthritis score 80% at day 23 (Fig. 6). Anti-type II collagen antibody level in serum was reduced by treatment with KF20444 (62% inhibition at day 18: $P = 0.02$). Brequinar (3 mg/kg) also reduced the arthritis score, and suppressed serum anti-type II collagen antibody level (60% inhibition at day 18: $P = 0.03$).

Table 3. Dose response analyses of KF20444 and brequinar on rat CIA[a].

Treatment	Dose (mg/kg)	Incidence of arthritis[b]	Serum anti-type II collagen antibody[c]	Bone destruction score[d]
Normal	–	0/7	0.001 ± 0.001****	0.0 ± 0.0
Control	–	7/7	0.480 ± 0.064	11.0 ± 2.2
KF20444	0.3	6/7	0.440 ± 0.051	9.2 ± 2.0
	1	4/7	0.179 ± 0.038***++	1.7 ± 1.1**+
	3	0/7	0.004 ± 0.002****++	0.0 ± 0.0****
Brequinar	0.3	7/7	0.533 ± 0.035	14.0 ± 0.6
	1	7/7	0.464 ± 0.030	6.6 ± 1.5
	3	3/7	0.132 ± 0.028****	0.0 ± 0.0****

[a] Results are shown as the mean ± SE. [b] The incidence of arthritis was examined at day 18. [c] Anti-type II collagen antibody level in serum at day 18 was measured by ELISA and expressed as absorbance at 490 nm. [d] Radiographs were taken on day 18.
; P < 0.01, *; P < 0.001, ****; P < 0.0001 compared with control group. +; P < 0.05, ++; P < 0.01 compared with equal dose of brequinar treated group.

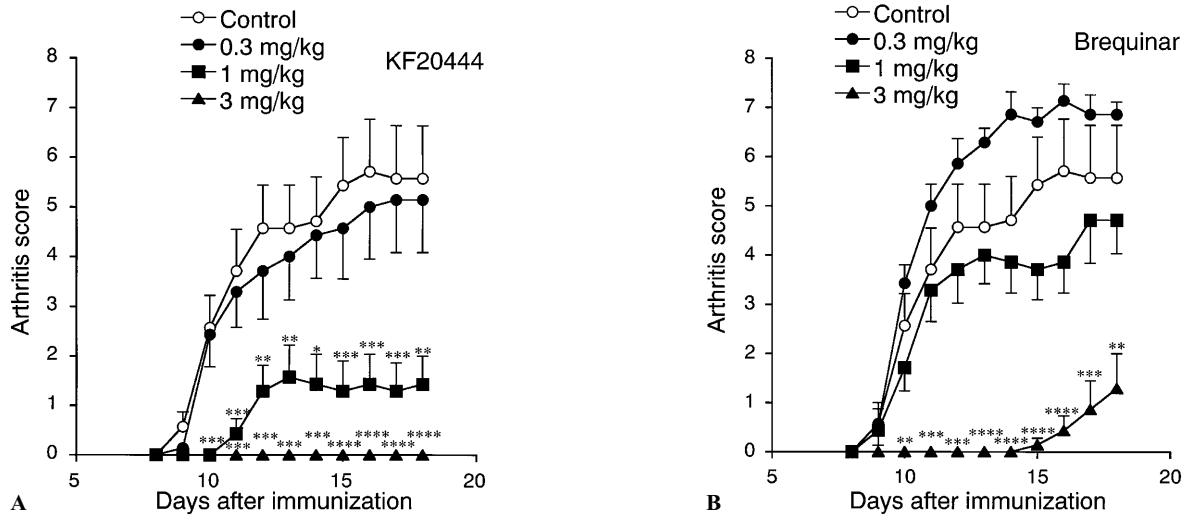


Fig. 5. Dose dependency of KF20444 and brequinar on the arthritis score. Each compound was administered orally 5 times per week from day 0 to day 17 in rat CIA. The arthritis score of both hind paws was examined. Vehicle (control) or 0.3 mg/kg, 1 mg/kg, 3 mg/kg of KF20444 were administered (**A**). Vehicle (control) or 0.3 mg/kg, 1 mg/kg, 3 mg/kg of brequinar were administered (**B**). Results are shown as the mean ± SE (N = 7). *; P < 0.05, **; P < 0.01, ***; P < 0.001, ****; P < 0.0001 compared with control group.

Effects of KF20444 and brequinar on the DHO-DHase activity

The activity of DHO-DHase partially purified from rat liver was evaluated; the apparent K_m value of the substrate (L-DHO) was 28.6 ± 1.3 μM (mean ± SE in six experiments) and the V_{max} was 263 ± 29 nmol/h/mg protein, determined from a Lineweaver-Burk plot. Both brequinar and KF20444 inhibited DHO-DHase activity in a concentration dependent manner (data not shown). Both compounds exhibited mixed competitive and noncompetitive inhibition. The apparent K_i values of KF20444 and brequinar, were 8.5 ± 3.2 nM and 25.3 ± 5.3 nM, respectively (Table 4). The apparent K_i' of KF20444 and brequinar were 0.81 ± 0.08 nM and 1.40 ± 0.21 nM, respectively.

Inhibitory activity of KF20444 and brequinar against Lck tyrosine kinase

In this study, the activity of Lck from bovine thymus was measured using synthetic peptides. Neither KF20444 nor brequinar inhibited Lck over the concentration range 1 to 200 μM (data not shown). However, staurosporine, which is a non-selective protein kinase inhibitor [24], inhibited the phosphorylation of the synthetic peptides by Lck with an IC_{50} value of 0.07 μM.

Effects of KF20444 and A77 1726, an active metabolite of leflunomide, on cell cycle progression in Jurkat cells

KF20444 inhibited the proliferation of Jurkat cells, a human T-cell line, with an IC_{50} value of 0.16 μM (data not shown).

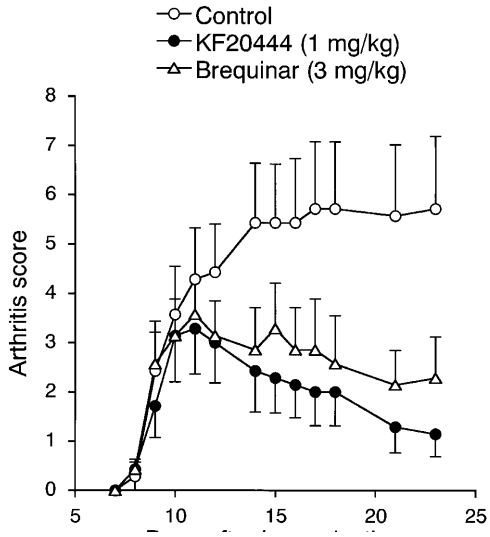


Fig. 6. Effects of KF20444 and brequinar on arthritis score when drugs were administered from day 7 in rat CIA. Each drug was administered orally 5 times per week from day 7 to day 22. Vehicle (control), KF20444 (1 mg/kg), or brequinar (3 mg/kg) were administered. Results are shown as the mean ± SE (N = 7).

Jurkat cells treated with KF20444 for 6 h induced cell cycle arrest at the S-phase and entry into the G_2- and M-phase was inhibited (Fig. 7A); the minimum effective concentration of KF20444 was 0.03 µM. The active metabolite of leflunomide, namely A771726, also showed similar results (Fig. 7B). These results suggested that the anti-proliferative effect of KF20444 was due to cell cycle arrest that might occur via the same mechanism as for leflunomide.

Discussion

In this study, oral administration of KF20444 at 3 mg/kg before immunization of type II collagen completely prevented the development of CIA in rats. When assessing the clinical potential of drugs for the treatment of patients with RA, it is probably more relevant to consider the effects of compounds on established disease. Therefore, it was encouraging to note that the arthritis score was reduced when rats were administered KF20444 from 7 days after immunization of type II collagen in CIA model.

Although brequinar was also effective in a prophylactic experiment, it was less potent than KF20444. Rats treated with brequinar at 3 mg/kg developed arthritis at a late phase of CIA, while rats treated with KF20444 at an equal dose did not develop arthritis. Prednisolone and naproxen also suppressed the arthritis score, but did not inhibit the production of anti-type II collagen antibodies. In contrast, KF20444 strongly inhibited the production of anti-type II collagen antibodies. Previously we reported that KF20444 inhibited both T cell dependent and independent antibody production [1].

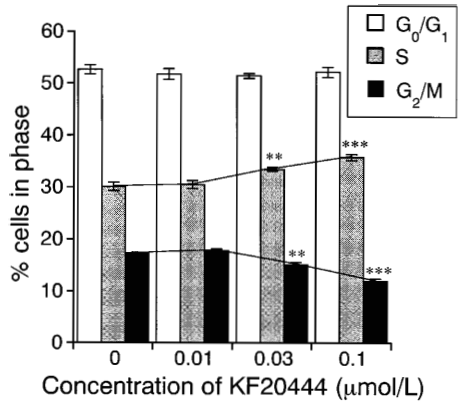
In this study, it was discovered that KF20444 inhibited DHO-DHase and that this affect was 3 times more potent than that of brequinar. The apparent K_i value of brequinar sodium previously reported was between 5 to 240 nM [8, 27, 28], depending on the source of the enzyme. The potency of KF20444 and brequinar was correlated with the efficacy in vivo. The immunosuppressive efficacy of brequinar sodium has been widely accepted to be due to the inhibition of DHO-DHase. There are 4 possible mechanisms for inhibition of DHO-DHase activity; (i) competitive inhibition at the active site of DHO-DHase, e.g. orotic acid and its analogs [29]; (ii) non-competitive inhibition of the electron transport system, e.g. some naphthoquinones [30]; (iii) mixed competitive and noncompetitive inhibition, e.g. brequinar sodium [27]; (iv) non-specific inhibition by electron carriers, e.g. cyanid and 2,4,-dinitrophenol [31]. The apparent K_i' value of KF20444 is lower than the respective apparent K_i value, which probably infers that KF20444 is a mixed type inhibitor against DHO-DHase.

Recently, Xu and coworkers [10] observed that the anti-proliferative and immunosuppressive effects of a high con-

Table 4. Kinetics parameters of KF20444 and brequinar on DHO-DH activity in rat liver mitochondria[a].

	Apparent K_i (nM)	Apparent K_i' (nM)
KF20444	8.5 ± 3.2	0.81 ± 0.08
Brequinar	25.3 ± 5.3	1.40 ± 0.21

[a] Results are shown as the mean ± SE.



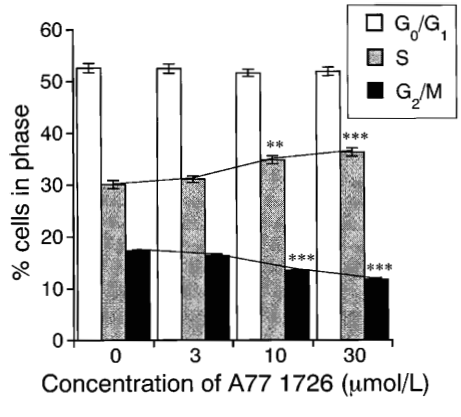


Fig. 7. Effects of KF20444 and leflunomide's active metabolite, A77 1726 on cell-cycle progression in Jurkat cells. Jurkat cells were treated with KF20444 (**A**) or A77 1726 (**B**) for 6 h and then analyzed on a flow cytometer. Results are shown as the mean ± SE (N = 3). **; P < 0.01, ***; P < 0.001 compared with vehicle treated group.

centration of brequinar sodium were not compensated by addition of uridine, and that brequinar sodium also inhibited purified Lck and Fyn at high concentrations (> 50 μM). Therefore, we studied the effects of KF20444 on Lck activity to ascertain whether inhibition of tyrosine phosphorylation could explain the anti-arthritic effects of the compound. However, we did not detect the inhibition of Lck by either KF20444 or brequinar up to 200 μM. The discrepancy between the present data and that reported by Xu et al. [10] may be attributable to the use of different species of Lck or different substrates. We used bovine Lck and evaluated the phosphorylation of a synthetic peptide, whereas Xu et al. used mouse Lck and evaluated the autophosphorylation of Lck or the phosphorylation of histone 2B [10]. However, even if KF20444 or brequinar inhibit Lck in vitro, this effect may not contribute to the suppression of CIA in vivo, because the plasma concentration of KF20444 after administration to rats does not reach such a high concentration (peak plasma concentration at 3 mg/kg p.o. = 6.25 ± 0.05 μM).

A DHO-DHase inhibitor, leflunomide, is an effective and safe treatment for patients with active RA [15]. The anti-inflammatory and anti-immunosuppressive properties of leflunomide are reported to derive from the inhibition of DHO-DHase [32–34] and tyrosine kinases [35]. Inhibition of DHO-DHase inhibition depletes the orotate and subsequently decreases uridine monophosphate [33, 35]. As a critical level of uridine monophosphate (ribonucleotides) is required to stabilize p53 [36], a lower level of uridine monophosphate activates p53 and subsequently cells are arrested in the G_1-phase of the cell cycle [12, 26, 37]. The effects of KF20444 on the cell cycle were similar to that of an active metabolite of leflunomide, A77 1726. These results suggest that KF20444 may also activate p53. Part of the immunosuppressive effect of leflunomide on the MRL/lpr mice is explained by the inhibition of the tyrosine kinases [35]. In the present study, KF20444 did not inhibit tyrosine kinases. Several alternative mechanisms have been proposed to explain the activities of leflunomide, including inhibition of the following: leukocyte adhesion [38], cyclooxygenase-2 [39], nuclear factor-κB (NF-B) [40] and IL-2 production [41]. Additional studies are required to examine the activities of KF20444 on these biological processes. We have previously reported the effect of KF20444 and leflunomide in the trinitrobenzenesulfonic acid (TNBS)-induced rat delayed type hypersensitivity (DTH) model [1]. Both KF20444 and leflunomide inhibited TNBS-induced paw edema with an ED_{50} of 0.60 mg/kg p.o. and 5.3 mg/kg p.o., respectively. The activity of leflunomide on DTH in our report is similar to that reported in a previous study [42]. Leflunomide has been reported to inhibit acute inflammation occurring in carrageenan-induced paw edema and ultraviolet-induced erythema [43]. However, KF20444 has no effect in carrageenan-induced paw edema at 3 mg/kg p.o. (data not shown). Therefore, the effect of leflunomide on acute inflammation is mediated via an activity not shared by KF20444; for example, it could be mediated via inhibition of tyrosine kinases. These data suggests that KF20444 is a more selective inhibitor of DHO-DHase.

In conclusion, KF20444 is a potent inhibitor of DHO-DHase and it completely prevents the development of CIA. Furthermore, it is effective after sub-therapeutic administration. These data suggest that KF20444 may have potential as a novel DMARD. Further studies on the anti-arthritic potential and toxicity associated with inhibition of pyrimidine synthesis are in progress prior to clinical study of KF20444.

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