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Combination benefit of a pyrimidylpiperazine derivative (Y-40138) and methotrexate in arthritic rats

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Abstract

Anti-tumor necrosis factor-α (TNFα) antibody in combination with methotrexate dramatically decreases joint destruction in rheumatoid arthritis. The aim of this study was to examine combined treatment with *N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide HCl (Y-40138) and methotrexate in rat adjuvant-induced arthritis. The increase in hindpaw volume and joint destruction was suppressed by single therapeutic administration (days 15–20) of Y-40138 (30 mg/kg, p.o.), but not by prophylactic administration (days 1–9). However, arthritic progression was suppressed by single prophylactic administration of methotrexate (0.3 mg/kg, p.o.), but not by therapeutic administration. Combined administration (days 10–20) of Y-40138 (0.3–1 mg/kg) and methotrexate (0.03 mg/kg) synergistically suppressed the increase in hindpaw volume and joint destruction. We concluded that Y-40138 in combination with methotrexate synergistically suppressed arthritic progression. These data suggest that combined treatment with Y-40138 and methotrexate may increase efficacy of therapy for rheumatoid arthritis.

Keywords: Y-40138; Methotrexate; Combination therapy; Adjuvant-induced arthritis; Joint destruction

1. Introduction

In rheumatoid arthritis, macrophages, T cells, plasma cells, dendritic cells, and fibroblasts are involved in the pathogenesis of synovitis that ultimately leads to tissue destruction. There is a large amount of tumor necrosis factor-α (TNFα) in synovial fluid and synovial tissue of patients with rheumatoid arthritis (Feldmann et al., 1996). And there is evidence that anti-TNFα antibodies (infliximab, adalimumab) (Maini et al., 1998; Weinblatt et al., 2003) and soluble TNFα receptor:Fc fusion proteins (etanercept) (Weinblatt et al., 1999) are clinically effective for the treatment of rheumatoid arthritis. Among many available anti-rheumatic drugs, TNFα blockers are currently considered the most effective for the treatment of rheumatoid arthritis (Newton and Decicco, 1999). Nevertheless, about 40% of patients with rheumatoid arthritis fail to respond to anti-TNFα treatment alone (Maini et al., 1998) thus there is increasing interest in combination therapy. Previously, we reported a novel pyrimidylpiperazine derivative Y-39041, *N*-[1-(4-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}phenyl)cyclopropyl] acetamide, which suppresses lipopolysaccharide-induced TNFα production in mice in vivo (Fukuda et al., 2001). Haddad et al. (2001) have investigated the mechanisms of action of Y-40138, a hydrochloride salt of Y-39041 in vitro. In alveolar epithelial cells, they reported that Y-40138 suppresses lipopolysaccharide-induced biosynthesis of TNFα and enhances biosynthesis of interleukin-10, which is an anti-inflammatory cytokine (De-Waal-Maleryt et al., 1991). In addition, Y-40138 blocks lipopolysaccharide-induced nuclear activation of nuclear factor-κB, and ameliorates degradation of inhibitory-κB alpha. These

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findings indicate that Y-40138 is a dual regulator suppressing TNFα production and augmenting interleukin-10 production, and its regulation is mediated through the inhibitory-κB alpha/nuclear factor-κB signal transduction pathway.

As a number of disease-modifying anti-rheumatic drugs often have side effects at high doses and/or during long-term administration, increased efficacy without increased toxicity has also been expected for combination therapy. Methotrexate is frequently used for the treatment of rheumatoid arthritis for 10 or more years. In clinical study, infliximab or etanercept has been used in combination with methotrexate for the treatment of rheumatoid arthritis to produce greater efficacy (Maini et al., 1998; Weinblatt et al., 1999). In collagen-induced arthritis, combined administration of anti-TNFα antibody and disease-modifying anti-rheumatic drugs, such as cyclosporin, increases efficacy compared to each agent alone (Williams et al., 1999).

The aim of the present study was to examine the combined effect of Y-40138 and methotrexate on the progression of rat adjuvant-induced arthritis. The progression of hindpaw inflammation and joint destruction was suppressed by single therapeutic administration of Y-40138, but not by prophylactic administration. On the other hand, single prophylactic administration of methotrexate almost completely suppressed the progression of hindpaw inflammation and joint destruction, but therapeutic administration did not. Therefore, it was expected that combined treatment with Y-40138 and methotrexate would increase efficacy in rat adjuvant-induced arthritis, since each agent alone can suppress arthritis at a completely different stage in this arthritic model.

It is necessary to have a weak anti-arthritic effect of each agent in order to examine increased efficacy by the combined treatment in rats. Administration of methotrexate to rats from day 1 to day 14 shows a strong anti-arthritic effect, dose-dependently in a narrow dose range (Bendele et al., 1999). They reported that methotrexate (0.3 mg/kg, p.o.), administered from day 5 to day 9 (prophylactic administration) and from day 15 to day 18 (therapeutic administration), has a weak anti-arthritic effect. On the other hand, methotrexate (0.1–1 mg/kg, p.o.) from day 15 to day 24 (therapeutic administration) does not suppress, or only slightly suppresses, paw inflammation of rats (Sakuma et al., 2001). It may be difficult to determine the methotrexate dose that will have a weak anti-arthritic effect during administration

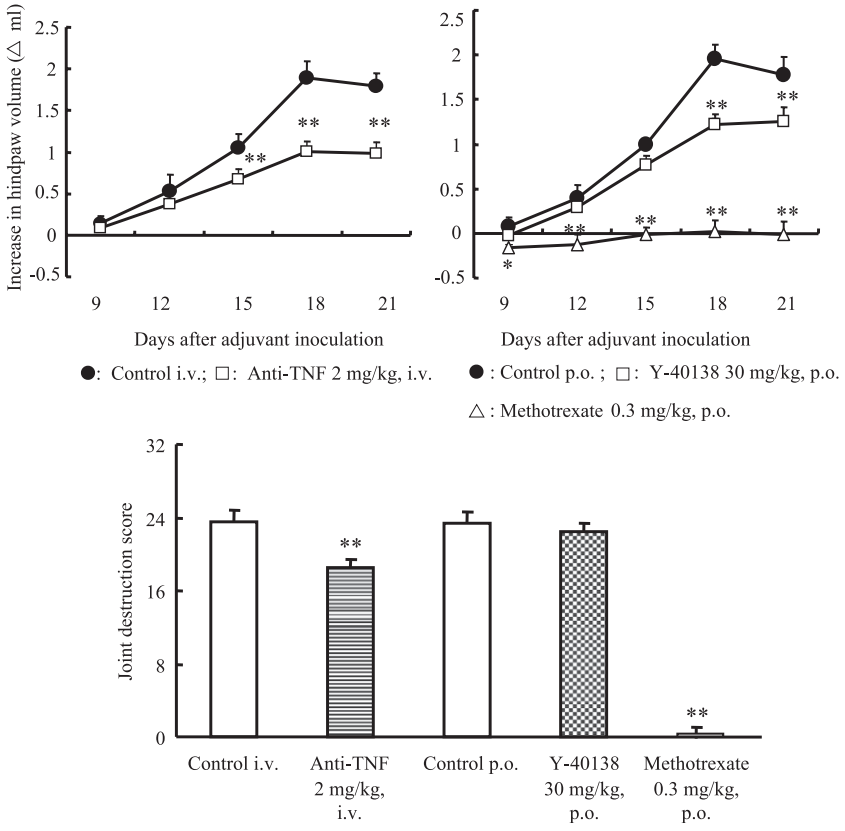


Fig. 1. Prophylactic effects of Y-40138, methotrexate and anti-murine TNFα antibody (anti-TNF) on increase in hindpaw volume and joint destruction in adjuvant-induced arthritic rats. Each agent was administered once a day from day 1 to day 9 after adjuvant inoculation with *M. butyricum*. Hindpaw volume was represented as the increase in the total volumes of right and left hindpaws from day 0 to each measurement date. Joint destruction was scored at day 21. Data are shown as the mean±S.E.M. (N=8). *P<0.05; **P<0.01, compared to control (Student's t-test).

periods from day 1 to day 14 or from day 1 to day 20. We assumed that a weak anti-arthritic effect of methotrexate was more easily determined by adjusting the administration period rather than the administration dosage.

In this study, we set the administration period from day 10 to day 20, which contained the duration of both semi-prophylactic and therapeutic administrations. As expected, Y-40138 or methotrexate administered from day 10 to day 20 showed a weak anti-arthritic effect. The combined treatment with a lower dose of Y-40138 and methotrexate synergistically suppressed the increase in hindpaw inflammation and joint destruction in arthritic rats. These results suggest that combined treatment with Y-40138 and methotrexate may increase efficacy of the treatment for patients with rheumatoid arthritis who have an insufficient response to methotrexate alone.

2. Materials and methods

2.1. Animals

Male specific pathogen-free LEW/Sea rats were purchased from Seac Yoshitomi (Fukuoka, Japan). Rats were housed under conditions of controlled temperature (23±3 °C) and 12h illumination cycles for at least 5 days before experiments. Animals were used at 6–7 weeks of age. All experiments were approved by the Animal Ethical Committee of Mitsubishi Pharma and performed in accordance with guidelines of the Japanese Pharmacological Society.

2.2. Compounds

Y-40138 was synthesized at Mitsubishi Pharma. Methotrexate injection was purchased from Wyeth Lederle Japan (Tokyo, Japan). Anti-murine TNFα antibody was purchased from Genzyme Techne (Cambridge, MA). Y-40138 and methotrexate injection were dissolved and diluted by 0.5% hydroxypropylmethylcellulose solution. Anti-murine TNFα antibody was dissolved in phosphate buffered saline.

2.3. Evaluation of arthritis

To induce arthritis, 0.5 mg of heat killed *Mycobacterium butyricum* (Difco, Detroit, MI) in 0.1 ml mineral oil was inoculated subcutaneously at the base of the tail under ether anesthesia at day 0. Each agent was

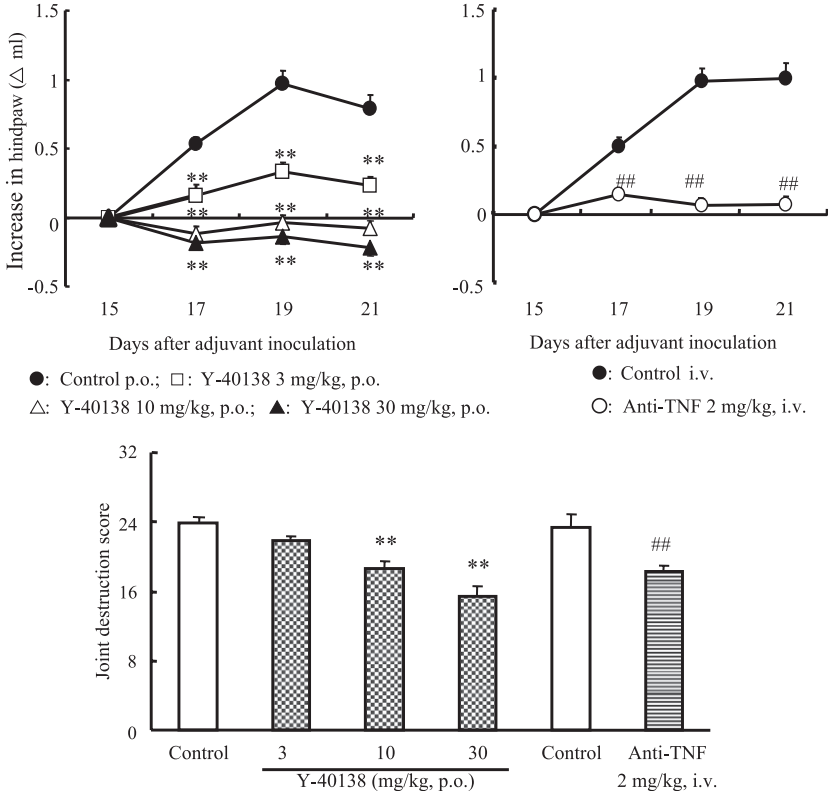


Fig. 2. Therapeutic effects of Y-40138 and anti-murine TNFα antibody (anti-TNF) on increase in hindpaw volume and joint destruction in adjuvant-induced arthritic rats. Each agent was administered once a day from day 15 to day 20 after adjuvant inoculation. Hindpaw volume was represented as the increase in the total volumes of right and left hindpaws from day 15 to each measurement date. Data are shown as the mean±S.E.M. (N=8). **P<0.01, compared to control (Dunnett's test). ##P<0.01, compared to control (Student's t-test).

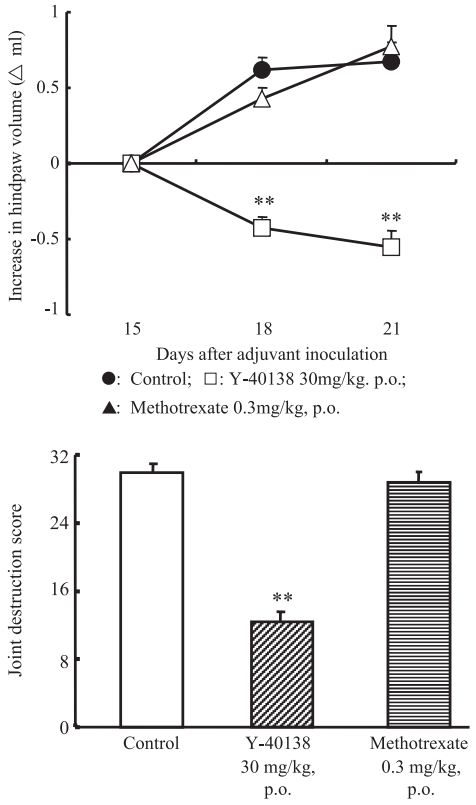


Fig. 3. Therapeutic effects of methotrexate and Y-40138 on joint destruction in adjuvant-induced arthritic rats. Each agent was administered once a day from day 15 to day 20 after adjuvant inoculation. Joint destruction was scored at day 21. Data are shown as the mean±S.E.M. (N=8). **P<0.01, compared to control (Student's t-test).

administered once a day according to its respective schedule. The volumes of both hindpaws were measured by aqueous plethysmography (Muromachi Kikai, Tokyo, Japan). Hindpaw volume was represented as the increase in total volumes of right and left hindpaws from day 0 (prophylactic treatment), from day 10 (combined treatment) or from day 15 (therapeutic treatment) to each measurement date. At day 21 animals were exsanguinated under ether anesthesia. Both hindpaws were excised and X-ray photography was done. Joint regions for evaluation were metatarsal bones, cuneiform bones, navicular and cuboid bones, shin, ankle and heel bones. Joint destruction grading used a score ranging from 0 to 4, and represented the sum of left and right scores (maximum 32). 0=no difference; 1=mild degree of calcic deposit; 2=calcic deposit (bone thickening); 3=mild joint destruction; 4=severe joint destruction.

2.4. Preparation of synoviocyte sample

Y-40138 was administered to arthritic rats once a day from day 10 to day 17. Knees were removed from between foot and thigh of each rat 2 h after final administration. Synoviocytes were separated from both knees and were added to 2 ml Isogen (Nippongene, Tokyo, Japan) on ice. Synoviocytes were homogenized on ice with glass homogenizer and stored at −80 °C until use.

2.5. Evaluation of TNFα mRNA expression in synoviocytes

Rat TNF signaling gene MPCR kit (Biosource International, Camarillo, CA) was used according to the manufacturer's instructions. Contents of glyceraldehyde 3-phosphate dehydrogenase were measured to correct for total nucleic acid content. Total RNA was extracted from the synoviocyte sample using the chaotropic Trizol method followed by Isogen-chloroform extraction and isopropanol precipitation (Chomczynski, 1993). Complementary DNA was prepared by reverse transcriptase (Perkin Elmer, Boston, MA) according to the manufacturer's instructions. The conditions of complementary DNA synthesis were as follows: 1 cycle for

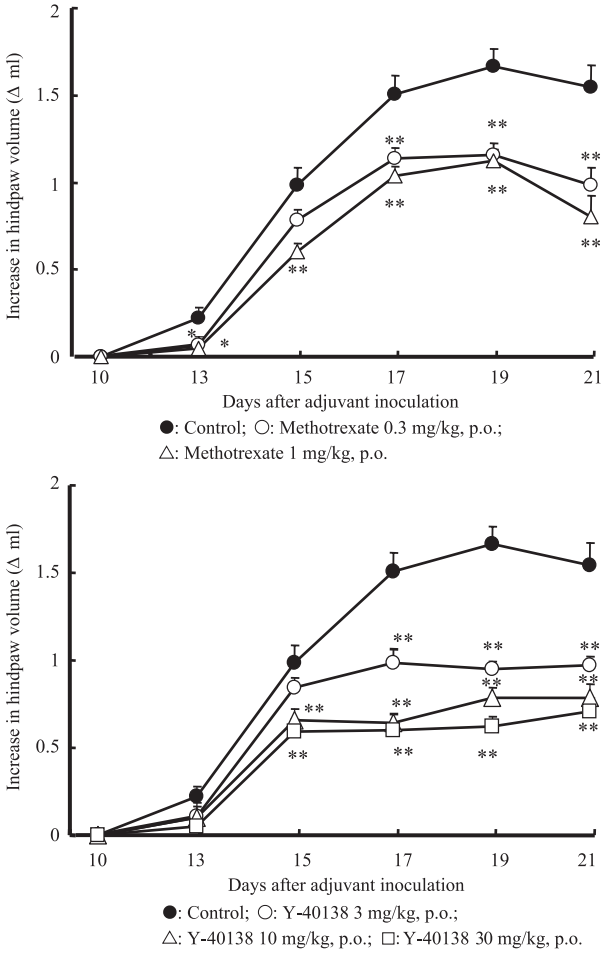


Fig. 4. Comparison of effects of Y-40138 and methotrexate on increase in hindpaw volume in adjuvant-induced arthritic rats. Each agent was administered once a day from day 10 to day 20 after adjuvant inoculation. Hindpaw volume was represented as the increase in the total volumes of right and left hindpaws from day 10 to each measurement date. Data are shown as the mean±S.E.M. (N=8). *P<0.05; **P<0.01, compared to control (Dunnett's test).

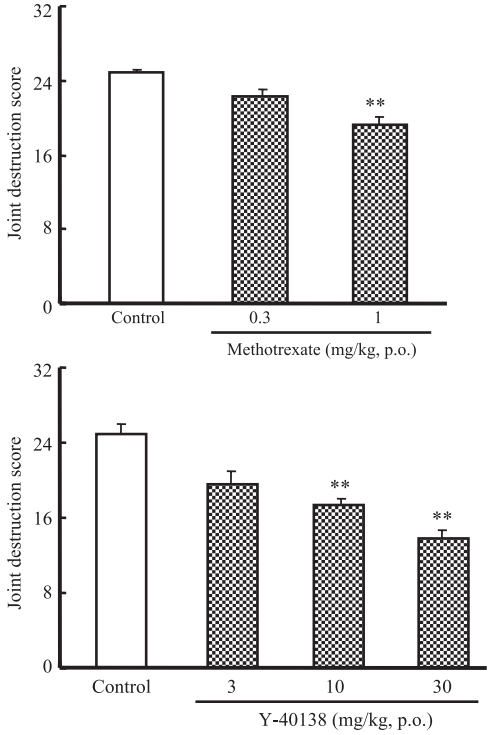


Fig. 5. Comparison of effects of Y-40138 and methotrexate on joint destruction in adjuvant-induced arthritic rats. Each agent was administered once a day from day 10 to day 20 after adjuvant inoculation. Joint destruction was scored at day 21. Data are shown as the mean±S.E.M. (N=8). **P<0.01, compared to control (Dunnett's test).

10 min at 30 °C, for 30 min at 42 °C and for 5 min at 99 °C. The conditions of polymerase chain reaction were as follows: 1 cycle for 1 min at 96 °C, 2 cycles for 1 min at 96 °C and for 4 min at 57 °C, 28 cycles for 1 min at 94 °C and for 2.5 min at 57 °C. The amplified polymerase chain reaction products were analyzed by electrophoresis in a 3% NuSieve agarose gel using a Tris–borate buffer (Takara Shuzo, Otsu, Japan) and visualized under UV illumination after ethidium bromide (Sigma, St. Louis, MO) staining. The band intensity was quantified using ChemiImager™ 400 (Alpha Innotech, San Leandro, CA). TNFα mRNA expression levels were presented as a ratio relative to glyceraldehyde 3-phosphate dehydrogenase mRNA expression.

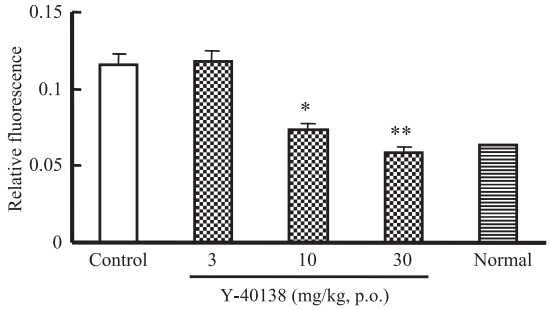
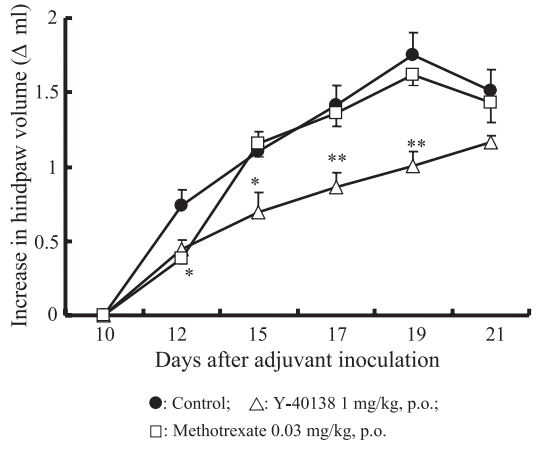


Fig. 6. Effect of Y-40138 on TNFα mRNA expression in synoviocytes of rats. Y-40138 was administered once a day from day 10 to day 17 after adjuvant inoculation. TNFα mRNA expression levels were measured at day 17 and were presented as a ratio relative to glyceraldehyde 3-phosphate dehydrogenase expression. Data are shown as mean±S.E.M. (except normal group; N=3) or a mean value (normal group: N=2). *P<0.05; **P<0.01, compared to control (Dunnett's test).

2.6. Statistical analysis

Data are presented as mean±S.E.M. or a mean value. Statistical significance of the increase in the total volumes of hindpaws and TNFα mRNA expression was determined using Dunnett's test or Student's t-test. Statistical significance of joint destruction score was determined using Wilcoxon's rank sum test, nonparametric Dunnett's test or Student's t-test. Differences were assessed with two-sided test, with an alpha level of 0.05.



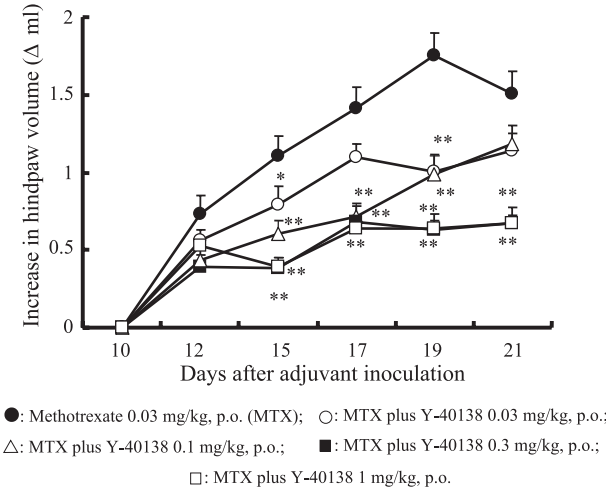


Fig. 7. Combined therapy of Y-40138 and methotrexate on increase in hindpaw volume in adjuvant-induced arthritic rats. Y-40138 (0.03–1 mg/kg, p.o.) and methotrexate (0.03 mg/kg, p.o.) were administered once a day from day 10 to day 20 after adjuvant inoculation. Hindpaw volume was represented as the increase in the total volumes of right and left hindpaws from day 10 to each measurement date. Data are shown as the mean±S.E.M. (N=8). *P<0.05; **P<0.01, compared to control (Student's t-test).

Table 1
Analysis of combined effect on hindpaw volume in adjuvant-induced arthritic rats

Dose (mg/kg, p.o.)		Mean CI value	95% confidence limit	
Y-40138	Methotrexate		Lower	Upper
0.03	0.03	0.15700	0.033055	0.74574
0.1	0.03	0.31486	0.045403	2.18353
0.3	0.03	0.01452	0.002715	0.07769
1	0.03	0.02375	0.003409	0.16552

2.7. Analysis of combined effect

The increase in hindpaw volume and the joint destruction score were used for analysis. Analyses by the combination index (CI) method were performed using the statistical software package SAS. The mean CI value and 95% confidence limit were calculated from the values of the increased hindpaw volume and the joint destruction score at day 21. A CI value <1 indicated synergism, >1 indicated antagonism, and a CI value of 1 indicated additivity.

3. Results

3.1. Prophylactic effect of anti-murine TNFα antibody, methotrexate and Y-40138 and on arthritis in rats

Anti-murine TNFα antibody (2 mg/kg, i.v.), methotrexate (0.3 mg/kg, p.o.) and Y-40138 (30 mg/kg, p.o.) were administered once a day from day 1 to day 9 after adjuvant inoculation. Anti-murine TNFα antibody suppressed the increase in hindpaw volume at days 15, 18 and 21 (Fig. 1). Methotrexate suppressed the increase in hindpaw volume significantly at days 9, 12, 15, 18, and 21. Y-40138 suppressed the increase in hindpaw volume at days 18 and 21. Anti-murine TNFα antibody lowered the joint destruction score significantly, but only slightly. Methotrexate lowered the joint destruction score significantly at day 21. Y-40138 did not lower the joint destruction score.

3.2. Therapeutic effect of Y-40138, anti-murine TNFα antibody and methotrexate on arthritis in rats

Y-40138 (3–30 mg/kg, p.o.), anti-murine TNFα antibody (2 mg/kg, i.v.) and methotrexate (0.3 mg/kg, p.o.) were administered from day 15 to day 20 after adjuvant inoculation. Y-40138 (3–30 mg/kg, p.o.) and anti-murine TNFα antibody suppressed the increase in hindpaw volume significantly at days 17, 19 and 21 (Fig. 2). Y-40138 (10 and 30 mg/kg, p.o.) and anti-murine TNFα antibody significantly lowered the joint destruction score at day 21. Methotrexate did not suppress the increase in hindpaw volume at days 18 and 21, and did not lower the joint destruction score (Fig. 3). As a positive control, Y-40138 (30 mg/kg, p.o.) significantly suppressed the increase in hindpaw volume at days 18 and 21, and lowered the joint destruction score.

3.3. Comparison of effect of methotrexate and Y-40138 on arthritis in rats

Methotrexate (0.3 and 1 mg/kg, p.o.) and Y-40138 (3–30 mg/kg, p.o.) were administered from day 10 to day 20 after adjuvant inoculation. Methotrexate at 1 mg/kg, p.o. suppressed the increase in hindpaw volume significantly at days 13, 15, 17, 19, and 21, and at 0.3 mg/kg, p.o. also suppressed the increase in hindpaw volume at days 13, 17, 19, and 21 (Fig. 4). Y-40138 (10 and 30 mg/kg, p.o.) suppressed the increase in hindpaw volume at days 15, 17,

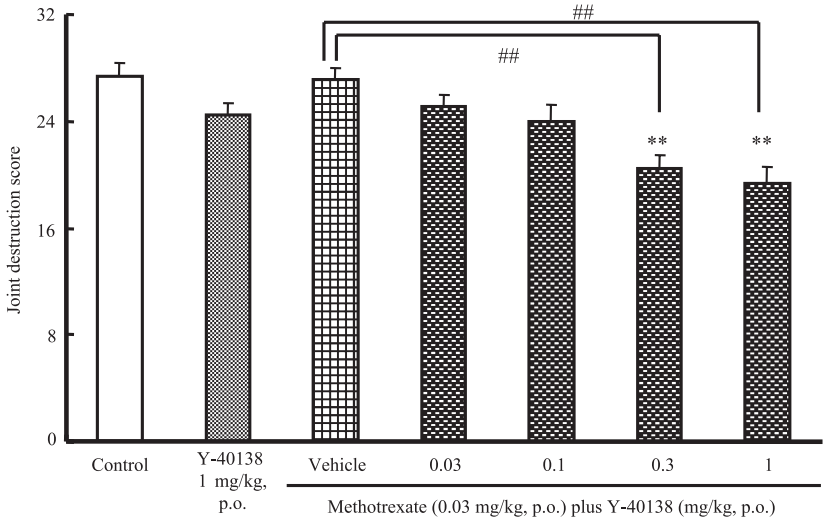


Fig. 8. Combined therapy of Y-40138 and methotrexate on joint destruction in adjuvant-induced arthritic rats. Y-40138 (0.03–1 mg/kg, p.o.) and methotrexate (0.03 mg/kg, p.o.) were administered once a day from day 10 to day 20 after adjuvant inoculation. Joint destruction was scored at day 21. Data are shown as the mean±S.E.M. (N=8). **P<0.01, compared to control (Dunnett's test). ##P<0.01, compared to methotrexate group alone (Student's t-test).

19 and 21, and at 3 mg/kg, p.o. also suppressed the increase in hindpaw volume at days 17, 19 and 21. Methotrexate at 1 mg/kg, p.o. lowered the joint destruction score significantly at day 21, but not at 0.3 mg/kg (Fig. 5). Y-40138 at doses of 10 and 30 mg/kg, p.o. lowered the joint destruction score significantly, but not at 3 mg/kg.

3.4. Effect of Y-40138 on TNFα mRNA expression in synoviocytes of rats

The mRNA expression of TNFα in synoviocytes of rats was determined at day 17 after adjuvant inoculation (Fig. 6). Y-40138 at 10 and 30 mg/kg, p.o. decreased TNFα mRNA expression significantly, but not at 3 mg/kg.

3.5. Combined effect of Y-40138 and methotrexate on arthritis in rats

Y-40138 (0.03–1 mg/kg, p.o.) and methotrexate (0.03 mg/kg, p.o.) were administered from day 10 to day 20 after adjuvant inoculation. Single administration of Y-40138 (1 mg/kg, p.o.) suppressed the increase in hindpaw volume significantly at days 15, 17 and 19, but not at days 12 and 21 (Fig. 7). Single administration of methotrexate suppressed the increase in hindpaw volume only at day 12, but not at days 15, 17, 19 and 21. The combined treatment with Y-40138 (0.03 mg/kg, p.o) and methotrexate suppressed the increase in hindpaw volume significantly at days 15 and 19 compared to the control group. And the combined treatment with Y-40138 (0.1 mg/kg, p.o) and methotrexate suppressed the increase in hindpaw volume significantly at days 15, 17 and 19, but not at day 21. The combined treatment with Y-40138 (0.3 and 1 mg/kg, p.o.) and methotrexate suppressed the increase in hindpaw volume significantly at days 15, 17, 19 and 21. The mean CI value from analysis of the combined effect on hindpaw volume at day 21 was less than 1.0 in all groups (Table 1).

Single administration of Y-40138 (1 mg/kg, p.o.) did not lower the joint destruction score at day 21 (Fig. 8). The combined treatment with Y-40138 (0.3 and 1 mg/kg, p.o.) and methotrexate lowered the joint destruction score significantly compared to the control group, and to the single treatment with methotrexate group. The mean CI value from analysis of the combined effect on the joint destruction score was less than 1.0 in all groups (Table 2). Therefore, the suppressive effects of the combined treatment with Y-40138 (0.3 and 1 mg/kg, p.o.) and methotrexate (0.03 mg/kg, p.o.) on the increase in hindpaw volume and joint destruction were considered to be synergistic.

Table 2
Analysis of combined effect on joint destruction in adjuvant-induced arthritic rats

Dose (mg/kg, p.o.)		Mean CI value	95% confidence limit	
Y-40138	Methotrexate		Lower	Upper
0.03	0.03	0.17022	0.035134	0.82469
0.1	0.03	0.15059	0.043475	0.52162
0.3	0.03	0.06967	0.032438	0.14964
1	0.03	0.12382	0.058176	0.26352

4. Discussion

The increased levels of TNFα in arthritic tissue frequently correlate with severity of rheumatoid arthritis (Feldmann et al., 1996). Rat adjuvant-induced arthritis has often been used to study the mechanisms of action and preventive effects of a number of disease-modifying anti-rheumatic drugs. The increase in TNFα levels in paw tissues of adjuvant-induced arthritic rats has been reported to correlate with severity of arthritis during a period of 15–21 days after adjuvant inoculation, and not at day 4 and at day 10 (Magari et al., 2003). Therefore, it is suggested that TNFα production may be increased in arthritic rats during the 15–21 days after adjuvant inoculation, and may not be stimulated until day 10. If excessive TNFα production could be blocked in the inflamed arthritic joint, it is supposed that progression of arthritis could be markedly suppressed. In order to confirm the participation of TNFα in rat arthritis, anti-murine TNFα antibody was administered during days 1–9, or 15–20 days after adjuvant inoculation. As expected, therapeutic administration (days 15–20) of anti-murine TNFα antibody had a strong suppressive effect, but prophylactic treatment (days 1–9) had a weak or no suppressive effect. These results suggest that TNFα produces a marked enhancement in arthritic rats during days 15–20, but not during days 1–9.

It is reported that methotrexate has species differences in oral absorption between rats and humans (Ahern et al., 1988; Kuroda et al., 2000). Reportedly, bioavailability in oral administration of 0.5 mg/kg methotrexate to rats is about 10%. The low bioavailability in rats is mainly a result of incomplete absorption, and greatly differs from the 73% in humans. Therefore, the methotrexate dose to rats needs to be larger than that to humans. In this study we used almost the same methotrexate administration dose and administration frequency already reported in rats (Magari et al., 2003; Sakuma et al., 2001). Prophylactic administration of methotrexate (days 1–9) almost completely suppressed arthritic progression in rats. On the other hand, therapeutic administration of methotrexate (days 15–20) had no suppressive effect on arthritic progression. This result suggests that methotrexate is not effective in all rheumatoid arthritis patients. Based on American College of Rheumatology 20 evaluation, methotrexate treatment for rheumatoid arthritis is not 100% effective. Methotrexate has no inhibitory effect on TNFα production from human peripheral blood mononuclear cells induced by lipopolysaccharide in vitro (Sakuma et al., 2000). Therefore, methotrexate may have an effect on rat arthritis through actions other than suppression of TNFα production. From these results and findings, we concluded that TNFα blocker suppresses

arthritis in the late stage (days 15–20), which is a TNFα-rich stage. In arthritic rats, we could not detect TNFα levels in inflamed paws from day 15 to day 20 (data not shown). This finding is inconsistent with a report by Magari et al. (2003). The reason for no detection in inflamed paws is unknown, but may be due to differences in sensitivity of enzyme linked immunosorbent assay.

Interestingly, therapeutic administration of Y-40138 had a dramatically strong inhibitory effect on arthritis in rats, while prophylactic administration of Y-40138 had no inhibitory effect. This indicates that Y-40138 may suppress arthritic progression through suppression of TNFα production. Next, we investigated whether the anti-arthritic effect of Y-40138 is dependent on suppression of TNFα production, since Y-40138 strongly suppresses arthritis at the late stage. Y-40138 significantly decreased TNFα mRNA expression in synoviocytes of knee joints in arthritic rats. This finding is supported by the fact that Y-39041 (freebase of Y-40138) suppresses lipopolysaccharide-induced TNFα production in arthritic rats (Hisadome et al., 2001). Y-40138 also suppressed lipopolysaccharide-induced TNFα production in arthritic rats (data not shown). The above results and findings suggest that Y-40138 can suppress the progression of arthritis through suppression of TNFα levels in inflamed sites. In the future, it will be necessary to investigate in detail the influence of Y-40138 on signal transduction pathway. As well as Y-40138, therapeutic treatment (days 11–17), but not prophylactic treatment (days 0–6), with liposomally conjugated methotrexate markedly suppresses arthritis in rats (Williams et al., 1994a). As liposomally conjugated methotrexate suppresses TNFα production from rat macrophages (Williams et al., 1994b,c), arthritis may be suppressed through the suppression of TNFα production. Therefore, anti-arthritic effect of Y-40138 and liposomally conjugated methotrexate on arthritis may be due to suppression of TNFα production in rat adjuvant-induced arthritis.

As a number of disease-modifying anti-rheumatic drugs in monotherapy often have unexpected side effects, combined treatment at lower doses may be necessary in order to expand the margin between efficacy and toxicity. The principal aim of this study was to examine the combined effect of lower dose Y-40138 and methotrexate on the progression of hindpaw inflammation and joint destruction in rats. It is expected that combined treatment with Y-40138 and methotrexate may increase efficacy compared to each agent alone, since each agent can suppress a completely different stage of the inflammatory process in arthritic rats. It is very important to determine the administration period of test agents in order to estimate the combined effect on arthritis. As stated, TNFα levels in arthritis increase in proportion to severity of arthritis during days 15–20 (Magari et al., 2003), and methotrexate, administered during both periods of days 5–9 and days 15–18, has a mild suppressive effect (Bendele et al., 1999). In order to obtain the mild suppressive effect of each agent, we set up an administration period (days 10–20) containing the duration of both the semi-prophylactic and therapeutic administrations. As expected, each agent administered from day 10 to day 20 showed a weak anti-arthritic effect. The combined treatment with low doses of Y-40138 and methotrexate synergistically suppressed the progression of hindpaw inflammation and joint destruction in rats. In arthritic rats, pegylated soluble TNFα receptor in combination with methotrexate has an additively suppressive effect on joint swelling (Bendele et al., 1999). A TNFα blocker in combination with low dose methotrexate may provide potentially synergistic benefits and hence have better disease modification with less risk of deleterious effects. In particular, an orally active TNFα blocker has been very useful in clinical studies. When Y-40138 (3 and 10 mg/kg, p.o.) was administered orally once a day for 26 weeks in rats and cynomolgus monkeys, Y-40138 was well tolerated and had good bioavailability (data not shown). Repeated administration of Y-40138 for 26 weeks did not directly cause liver damage in rats and monkeys. Repeated administration of methotrexate (0.1 mg/kg, p.o.) for 42 days shows liver injury in rats (Hall et al., 1991). The above findings suggest that toxic symptoms, especially liver damage, caused by repeated administration of Y-40138 and methotrexate may not overlap. Therefore, arthritis treatment with a combination of Y-40138 and methotrexate may offer better efficacy and lower side effects.

In summary, combined administration of Y-40138 and methotrexate synergistically suppressed arthritic progression in rats. This suggests that combined treatment with Y-40138 and methotrexate may have beneficial effects for the treatment of rheumatoid arthritis.

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