Identification of a CXCR4 antagonist, a T140 analog, as an anti-rheumatoid arthritis agent

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Abstract Several recent papers support the involvement of an interaction between stromal cell-derived factor-1 (SDF-1/CXCL12) and its receptor, chemokine receptor CXCR4, in memory T cell migration in the inflamed rheumatoid arthritis (RA) synovium. Analogs of the 14-mer peptide T140 were previously found to be specific CXCR4 antagonists that were characterized as not only HIV-entry inhibitors but also anti-cancer-metastatic agents. In this study, a T140 analog, 4F-benzoyl-TN14003, was proven to inhibit CXCL12-mediated migration of human Jurkat cells and mouse splenocyte in a dose-dependent manner in vitro (IC_{50} = 0.65 and 0.54 nM, respectively). Furthermore, slow release administration by subcutaneous injection (s.c.) of 4F-benzoyl-TN14003 using an Alzet osmotic pump significantly suppressed the delayed-type hypersensitivity response induced by sheep red blood cells in mice, and significantly ameliorated clinical severity in collagen-induced arthritis in mice. As such, T140 analogs might be attractive lead compounds for chemotherapy of RA.

Keywords: CXCR4 antagonist; Rheumatoid arthritis; T140; Collagen-induced arthritis; Delayed-type hypersensitivity

1. Introduction

Inflammatory cytokines, such as IL-1, IL-6, IFN-γ and tumor necrosis factor (TNF)-α, and activation markers play a central role in the chronic rheumatoid arthritis (RA) synovium [1]. Development of biological drugs targeting these cytokines, such as humanized monoclonal antibodies, has produced promising results in clinical therapy of RA patients. However, this therapy has not yet reached a stage of perfection, and development of other drugs with novel action mechanisms that are independent of the above cytokine's functions, is required for the improvement of RA chemotherapy. Intrinsically, RA is caused by the $CD4^+$ memory T cell accumulation in the inflamed synovium. Nanki et al. [2] reported that the memory T cells highly express a chemokine receptor CXCR4, and that the concentration of stromal cell-derived factor-1 (SDF-1/CXCL12), an endogenous ligand of CXCR4, is extremely high in the synovium of RA patients compared to controls. They also found that CXCL12 stimulates migration of the memory T cells and inhibits T cell apoptosis, suggesting that the CXCL12–CXCR4 interaction plays an important role in T cell accumulation in the RA synovium. Since chemokine CXCL12 [3–6] is independent of the inflammatory cytokines such as TNF-α, in terms of its expression and action, development of substances that inhibit the CXCL12–CXCR4 interaction might be promising as drugs. We have developed several CXCR4 antagonists, T22 (an 18-mer peptide) [7], T140 (a 14-mer) [8] and FC131 (a cyclic pentapeptide) [9]. These peptides have inhibitory activity against entry of T-cell line-tropic (X4-) HIV-1 into target cells [7,10,11] as well as against cancer metastasis and progression in breast cancer [12], pancreatic cancer [13,14], melanoma [15], acute lymphoblastic leukemia [16], small cell lung cancer [17], etc. In this study, we investigated whether a bio-stable T140 analog, 4F-benzoyl-TN14003 [18], shows anti-RA activity in the following experiments: inhibition assays of Jurkat cell/splenocyte migration mediated by CXCL12 in vitro, the delayed-type hypersensitivity (DTH) response induced by sheep red blood cells (SRBC) in vivo and collagen-induced arthritis (CIA) in vivo.

2. Material and methods

2.1. Material

4F-benzoyl-TN14003 [4-fluorobenzoyl–Arg–Arg–Nal–Cys–Tyr–Cit–Lys–D–Lys–Pro–Tyr–Arg–Cit–Cys–Arg–NH_2, a disulfide bond between Cys^4–Cys^{13}, Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline] was previously synthesized [18,19].

2.2. Migration assay of human Jurkat cells

Human Jurkat cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in RPMI-1640 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal calf serum (BioWhittaker).

Jurkat cells (2.5×10^6 cells/ml) were pre-incubated with various concentrations of 4F-benzoyl-TN14003 at 37 °C for 30 min. Then 200 μl of this suspension was placed into Transwell (Corning-Costar, Cambridge, MA, USA) culture inserts within 24-well culture plates containing 600 μl of assay medium (RPMI-1640/0.5% BSA/50 mmol/l HEPES) with 10 ng/ml human CXCL12 (Genzyme Techne, Minneapolis, MN, USA). The plates were incubated at 37 °C for 4 h in a humidified 5% CO_2 incubator. After the incubation, the culture inserts

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were removed and migrated cells from the inserts into the wells were counted using a Coulter counter. Percent migration was based on the total initial input per well.

2.3. Mouse splenocyte preparation and migration assay

Spleens were isolated from BALB/c mice (male, Charles River, Yokohama, Japan), and single-cell suspensions prepared from spleens were incubated in RBC lysing buffer (Immuno-Biological Laboratories, Fujioka, Japan), washed twice with PBS and then resuspended in assay medium.

Chemotaxis was measured in a 2.5-h Transwell migration assay as described above. Recombinant mouse CXCL12 (100 ng/ml, PeproTech, London, UK) was added to the wells in chemotaxis medium, and 1×10^7 cells were added to the Transwell inserts. Migrated cells were counted with a Coulter counter.

2.4. Inhibition assay of the mouse DTH response

SRBC were washed twice with saline, and then resuspended in saline. BALB/c mice (male 6 weeks, Charles River) were sensitized with a subcutaneous injection of 5×10^7 SRBC in 50 μl of saline into the left hind footpad. Five days later, mice were challenged by subcutaneously injecting 1×10^8 SRBC in 50 μl of saline into the right hind footpad. The thickness of the right hind paw was measured with a micrometer (Mitutoyo, CD-15B, Kawasaki, Japan) before treatment and 24 h after challenge. The DTH reaction was expressed as the swelling of the right footpad.

2.5. Induction and evaluation of CIA

Bovine type II collagen (CII, Collagen Research Center, Tokyo, Japan) was dissolved in 0.05 mol/l acetic acid to a concentration of 2 mg/ml, and the emulsion was prepared with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI, USA).

Six week-old male DBA/1J mice (Charles River) were injected intradermally at the base of the tail with a volume of 50 μl of the above emulsion. Twenty-one days after immunization, mice were given booster shots with the CII emulsion in the same manner. Following this injection, mice were evaluated for the incidence and the severity of arthritis, body weight and the thickness of the hind ankles, twice a week. At the end of the experimental period (2 weeks after the second injection), sera were obtained and the weights of the 4 limbs were measured.

The clinical severity of the arthritis was graded on a scale of 0–3 for each paw as follows: 0, normal; 1, swelling of one digit or mild swelling of the paw; 2, swelling of multiple digits or moderate swelling of the entire paw; 3, severe swelling of the entire paw. Each mouse could achieve a maximum score of 12.

2.6. Measurement of serum anti-bovine CII antibody

Levels of serum anti-bovine CII IgG2a antibody were measured using an ELISA assay. 96-well immunoplates were coated with bovine CII (10 μg/ml in PBS) and incubated at 4 °C overnight. Non-specific binding was blocked with PBS containing 10% FCS for 2 h. Mouse serum samples diluted with 10% FCS in PBS (1:1000) were added to the wells and incubated at room temperature for 2 h. Horseradish peroxidase-conjugated rat anti-mouse IgG2a antibody (1:1000 diluted, Zymed Laboratories, South San Francisco, CA, USA) was added and incubated for 1 h. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (DAKO A/S, Glostrup, Denmark) was added and allowed to react for 30 min. The reaction was stopped by the addition of an equal volume of 1 mol/l H_2SO_4, and the optical density at 450 nm was measured using a microplate reader (Labsystems MultiscanMS, Helsinki, Finland). For this ELISA, the wells were washed with PBS containing 0.1% Tween 20 before each step.

2.7. Drug administration

4F-benzoyl-TN14003 was dissolved in PBS, and subcutaneously administered using Alzet osmotic pumps (DURECT Corp., Cupertino, CA, USA), which were implanted dorsalaterally under the skin at the day before immunization (DTH) or the booster (CIA). In the experiment of DTH and CIA, #1007D pumps (delivering 4.8, 24 and 120 μg/day of 4F-benzoyl-TN14003 for 7 days) and # 2002 ones (delivering 120 μg of 4F-benzoyl-TN14003 daily for 14 days) were used, respectively. Indomethacin (1 mg/kg, Sigma Chemicals Co., St. Louis, MO, USA), methotrexate (3 mg/kg, Wako Pure Chemical Industries, Ltd, Osaka, Japan) and FK-506 (10 mg/kg, purified in Takeda Chemical Industries, Ltd, Osaka, Japan) were orally administered once daily for 2 weeks from the day of booster. These drugs were suspended in a 0.5% methylcellulose solution.

2.8. Statistical analysis

The values were represented as the means ± S.E. Statistical differences were determined using an analysis of variance (ANOVA, SAS software, version 6.1, SAS Institute, Cary, NC, USA). Results of DTH were assessed with the one-tailed Williams' test. A value of $P \leqslant 0.025$ was considered statistically significant. In the experiment involving CIA, unpaired or paired *t*-test were applied when only two values sets were compared. When data involved three or more groups, statistical analysis was performed using Dunnett's test. Clinical scores for each group were compared using the non-parametric Steel test or the Wilcoxon rank-sum test. The level of significance was defined as $P \leqslant 0.05$ and $P \leqslant 0.01$.

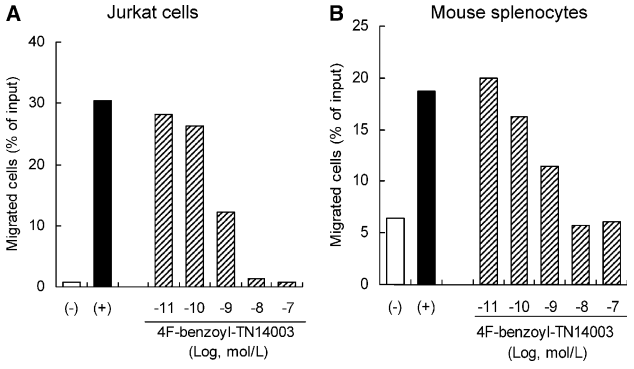

Fig. 1. Effects of 4F-benzoyl-TN14003 on CXCL12-induced migration of human Jurkat cells (A) and mouse splenocytes (B). Both cells were treated by CXCL12 (human CXCL12 10 ng/ml for Jurkat cells, mouse CXCL12 100 ng/ml for splenocytes) and various concentrations of 4F-benzoyl-TN14003. Control migrating cells in the absence and presence of CXCL12 are shown as (−) and (+), respectively. Data are expressed as means ($n = 2$).

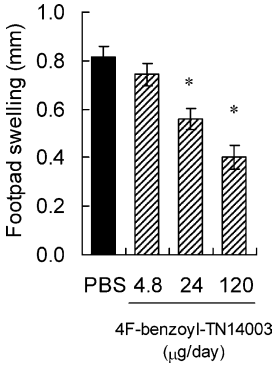

Fig. 2. Inhibition of the mouse DTH response by 4F-benzoyl-TN14003. The gain of thickness of right footpad by swelling 24 h after challenge was measured by a micrometer. PBS (control models) or 4F-benzoyl-TN14003 (4.8, 24 or 120 μg/day) was administered by s.c. injection using an Alzet pump from the day before immunization. Data are expressed as means ± S.E. ($n = 7$). * $P \leqslant 0.025$ (Williams' test).

3. Results

3.1. Inhibition of migration of human Jurkat cells and mouse splenocytes

Both human Jurkat cells and mouse splenocytes express CXCR4 on their surfaces [12,20]. CXCL12 (10 ng/ml = 1.1 nM for Jurkat cells, 100 ng/ml = 11 nM for splenocytes) dramatically enhanced the migration of Jurkat cells and splenocytes, as compared to control (absence of CXCL12, Fig. 1). 4F-benzoyl-TN14003 inhibited CXCL12-induced migration of these cells in a dose-dependent manner. At a concentration of 10 nM, 4F-benzoyl-TN14003 showed approximately 100% inhibition of cell migration induced by CXCL12 (1.1 nM for Jurkat cells, 11 nM for splenocytes). The IC_{50} values were determined to be 0.65 nM and 0.54 nM, respectively.

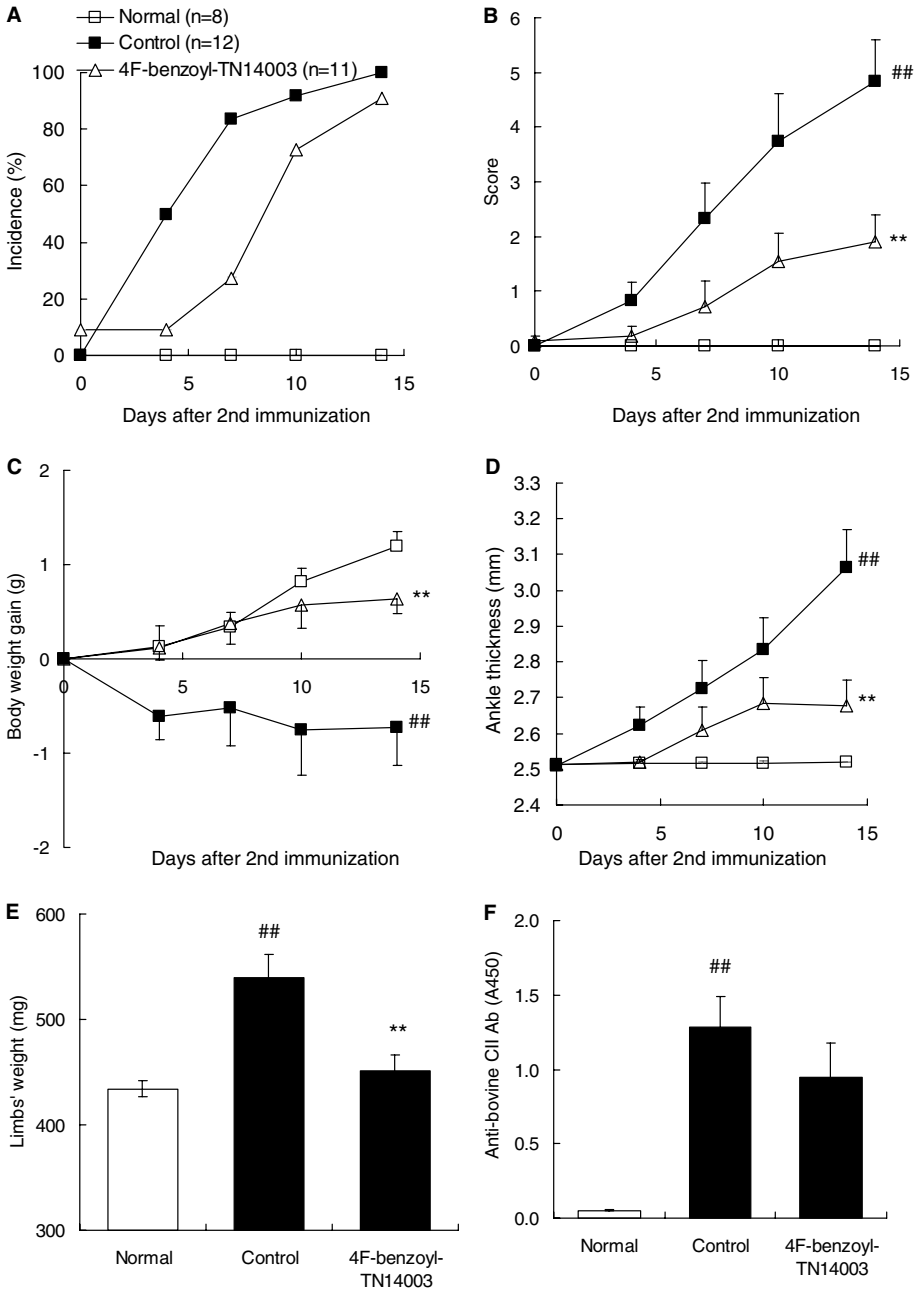

Fig. 3. Suppression of CIA in mice by 4F-benzoyl-TN14003. The incidence (A) and the score expressing the clinical severity (B) of arthritis were evaluated, and body weight (C) and the thickness of the hind ankles (D) were measured after the second immunization twice a week. The weights of 4 limbs were measured 2 weeks after the second immunization (E). Levels of anti-bovine CII IgG2a antibody in serum, which was obtained 2 weeks after the second immunization, were measured by ELISA (F). PBS (control models, $n = 12$) or 4F-benzoyl-TN14003 (120 μg/day, $n = 11$) was administered by s.c. injection using an Alzet pump from the day before the second immunization. In normal models ($n = 8$), mice were not immunized. Data are expressed as means ± S.E. $^{\#\#}P \leqslant 0.01$ (*t*-test): comparison with normal models, $^{**}P \leqslant 0.01$ (*t*-test): comparison with control models (Scores were compared by non-parametric Steel test).

3.2. Reduction of the DTH reaction in mice by subcutaneous administration of 4F-benzoyl-TN14003 using Alzet osmotic pumps

The SRBC-induced DTH reaction in mice was adopted as an in vivo experimental model of the cellular immune response for evaluation of 4F-benzoyl-TN14003 activity. The DTH reaction was estimated as the gain of right footpad thickness from swelling, 24 h after challenge. Seven mice were administered PBS as control models and showed significant footpad swelling (Fig. 2). Twenty one mice were administered 4F-benzoyl-TN14003 by s.c. injection using Alzet pumps (delivering 4.8, 24 and 120 μg/day, each for 7 mice) beginning from the day before immunization. Treated mice showed a dose-dependent suppression of swelling, as compared to control mice. The 24 and 120 μg daily injections showed inhibitory percentages of 31% and 51%, respectively. 4F-benzoyl-TN14003 significantly reduced the DTH reaction in mice.

3.3. Suppression of CIA in mice by subcutaneous administration of 4F-benzoyl-TN14003 using Alzet osmotic pumps

In the next phase, CIA was adopted to provide a second in vivo mouse model of this pathogenesis. During the 2 weeks after the CII emulsion booster, the following data were collected: the arthritis incidence, the scores expressing the severity, the body weight and thickness of the hind ankles were observed twice a week; the 4 limbs' weights and the serum anti-bovine CII antibodies were measured on the 14th day. Eleven mice were administered 4F-benzoyl-TN14003 by s.c. injection using Alzet pumps (delivering 120 μg/day), beginning from the day before the booster. Twelve mice were administered PBS as control models. Eight mice were not immunized as normal models. After observation for 14 days, 4F-benzoyl-TN14003-treated mice showed significant suppression of several symptoms of arthritis (score increase, body weight loss, ankle swelling and limbs' weight gain) as compared to the control mice that developed arthritis (Figs. 3 and 4). 4F-benzoyl-TN14003-treated mice also showed an apparent suppression of the extreme increase in levels of serum anti-bovine CII IgG2a antibody observed for the control group (Fig. 3F).

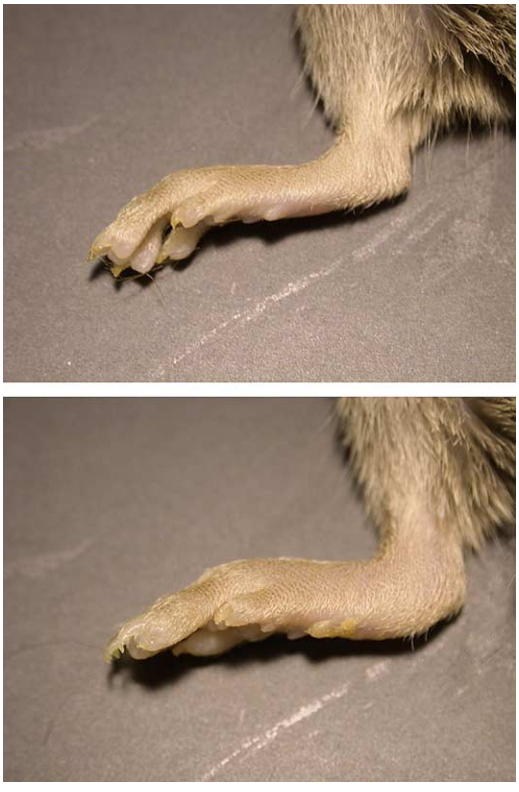

Fig. 4. Reduction of ankle swelling in mouse CIA by 4F-benzoyl-TN14003. Representative hind ankles of mice 2 weeks after the second immunization are shown. *Upper*, 4F-benzoyl-TN14003 was administered by s.c. injection using an Alzet pump from the day before the second immunization; *Lower*, PBS was administered as control models.

In a further comparative study, indomethacin [21] (1 mg/kg), methotrexate [22] (3 mg/kg) and FK-506 [23] (10 mg/kg), which are clinically used for treatment of RA patients or are known as anti-RA agents, were orally administered once daily for 2 weeks from the day of the booster (Fig. 5). These treated mice showed significant suppression of ankle swelling and limb weight gain, and an apparent suppression of marked increases in arthritis scores and anti-bovine CII antibody levels, remarkably similar to the results with 4F-benzoyl-TN14003. Therefore, 4F-benzoyl-TN14003 can be seen to possess inhibitory activity against RA symptoms, comparable to these known drugs.

4. Discussion

Chemokines constitute a chemotactic cytokine family that attract and induce migration of leukocytes, playing a fundamental role in the physiology of inflammation. CXCL12 is a chemokine that recognizes CXCR4. CXCR4 also has significant involvement in several pathological conditions, including AIDS [24], cancer [12–17,25] and RA [2]. The pathological importance of CXCR4 derives from its initial identification as the second receptor involved in the X4-HIV-1 entry into T cells [24]. According to recent reports, CXCL12 induces migration of several types of cancer cells via CXCR4 on their cell surfaces, causing cancer metastasis and progression [12–17,25]. Furthermore, the CXCL12/CXCR4 axis is thought to play an important role in the rheumatoid T cell accumulation in RA, as described in Section 1 [2]. Thus, CXCR4 represents an important therapeutic target for these diseases. We have previously developed several CXCR4 antagonists, T22, T140, 4F-benzoyl-TN14003 and FC131, which have strong anti-HIV-1 and anti-cancer metastatic activities, as described in Section 1 [7–17]. De Clercq's group reported another CXCR4 antagonist, AMD3100, which also has strong anti-HIV-1 activity [26]. Recently, he and his colleagues have found that AMD3100 inhibits autoimmune joint inflammation in IFN-γ receptor-deficient mice, and that AMD3100 interferes with cellular DTH reaction, but not with the humoral immune response to CII in CIA, as assessed by measuring anti-CII antibody levels [20]. In this study, we investigated whether 4F-benzoyl-TN14003 shows anti-RA activity by assessing its effects on humoral and cellular immunity.

We previously reported that 4F-benzoyl-TN14003 has strong binding capacity to CXCR4, as shown by inhibition of [^{125}I]-CXCL12 binding to CXCR4-expressing human Jurkat cells ($IC_{50} = 0.99$ nM) [12]. In this study, 4F-benzoyl-TN14003 was clearly shown to inhibit CXCL12-induced migration of Jurkat cells in a dose-dependent manner at subnanomolar levels, and also to inhibit mouse splenocyte migration induced by CXCL12. These results suggest that the activity of this com-

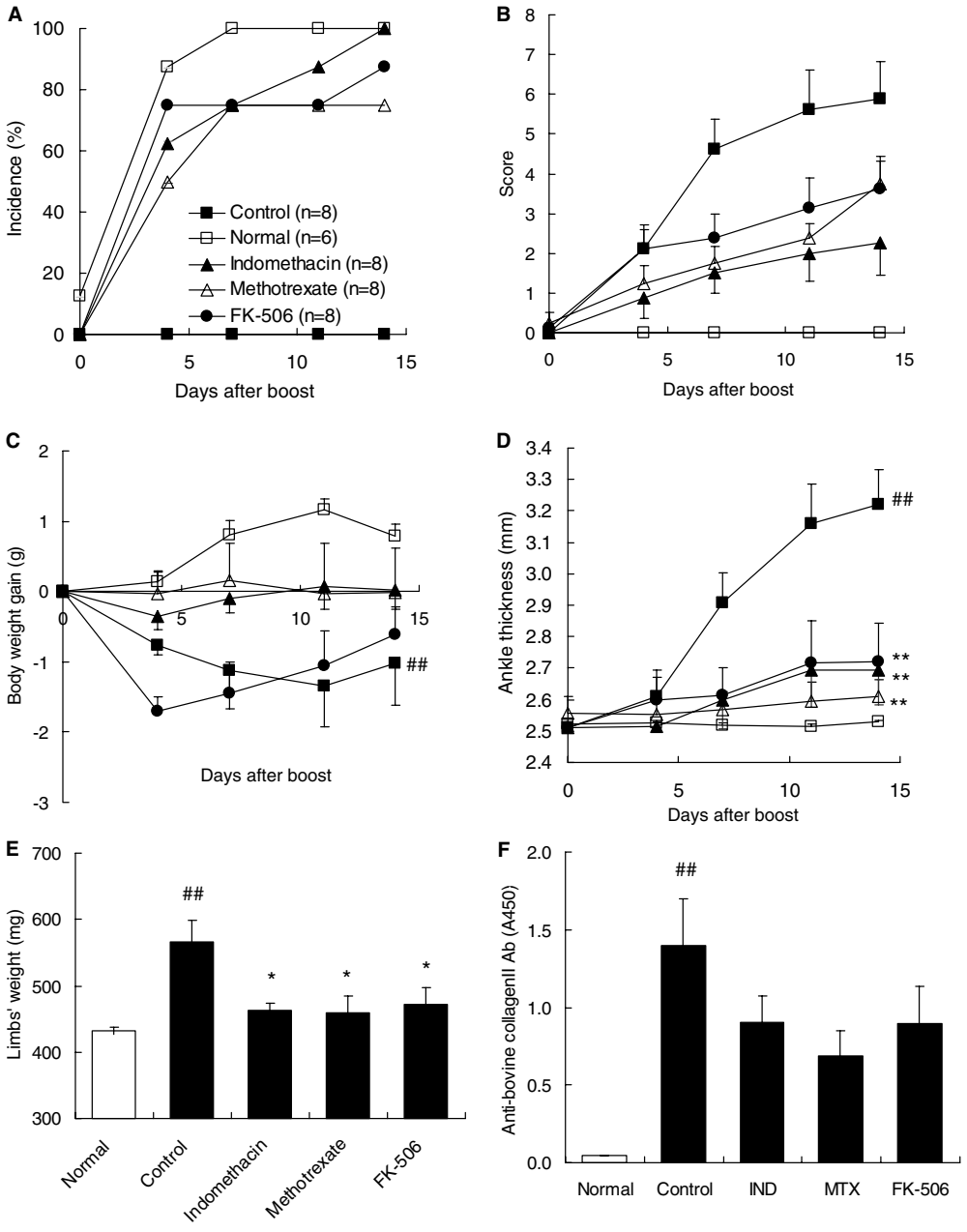

Fig. 5. Suppression of CIA in mice by known drugs. Efficacies of drugs were evaluated in the same way as in the experiments of Fig. 3 (A–F). PBS (control models, $n = 8$), indomethacin (1 mg/kg, $n = 8$), methotrexate (3 mg/kg, $n = 8$) or FK-506 (10 mg/kg, $n = 8$) was orally administered from the day of the second immunization once daily for 2 weeks. In normal models ($n = 6$), mice were not immunized. Data are expressed as means ± S.E. $^{\#\#}P \leqslant 0.01$ (t-test): comparison with normal models, $^{*}P \leqslant 0.05$ and $^{**}P \leqslant 0.01$ (Dunnett's test): comparison with control models.

pound is species independent, possibly due to the close homology of human and mouse CXCR4 [27]. Evaluation of the inhibitory activity of 4F-benzoyl-TN14003 against CXCL12-induced migration of CXCR4-expressing T cells suggests that 4F-benzoyl-TN14003 inhibits CXCL12-stimulated migration of memory T cells, thereby suppressing accumulation of T cells caused by inhibition of their apoptosis in the inflamed synovium of RA patients. Therefore, we investigated the inhibitory effects of 4F-benzoyl-TN14003 against RA using two mouse experimental models: the DTH response and CIA.

To evaluate the effect of 4F-benzoyl-TN14003 on cellular immunity, the DTH reactivity induced by SRBC was examined in mice. Subcutaneous administration of 4F-benzoyl-TN14003 using Alzet osmotic pumps significantly suppressed the footpad swelling. This indicates that 4F-benzoyl-TN14003 interferes with cellular immunity such as DTH, suggesting that CXCR4 might play an important role in cellular immunity.

Next, the activity of 4F-benzoyl-TN14003 was assessed in the CIA experiment, an RA animal model. The levels of several CIA symptoms were significantly lower in 4F-benzoyl-TN14003-treated mice than those in PBS-treated mice (arthritic control mice). Serum levels of the anti-CII IgG2a anti-body were also apparently lower in 4F-benzoyl-TN14003-treated mice. 4F-benzoyl-TN14003 therefore interferes with the humoral immune

response to CII, in contrast to AMD3100 [20]. The differences in the actions of AMD3100 and 4F-benzoyl-TN14003 in humoral CIA immunity might be explained by a difference in the binding sites of these CXCR4 antagonists [28] or by differences in the CIA models that were used.

In conclusion, the present results suggest that a bio-stable T140 analog, 4F-benzoyl-TN14003, inhibits the migration and accumulation of rheumatoid T cells through a specific binding to CXCR4 in competition with CXCL12. This compound interfered with cellular and humoral immune responses in experimental arthritis models in mice, and showed inhibitory effects against the CIA development at levels comparable to, or above those of known drugs. Since T140 is an inverse agonist, which has no CXCL12-like agonistic activity [29], it and its analogs have the potential of becoming promising agents for RA chemotherapy as well as for AIDS and cancer chemotherapy.

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