The Effect of TRK-530 on Experimental Arthritis in Mice

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TRK-530 is a newly synthesized diphosphonate derivative. We investigated the effect of TRK-530 on type II collagen-induced arthritis (CIA) in mice in comparison to that of prednisolone and indomethacin. TRK-530 at a dose of 25 mg/kg showed a tendency to inhibit CIA. TRK-530 at a dose of 50 mg/kg inhibited the development of the CIA in terms of the progression of footpad swelling, bone damage and histopathological changes. TRK-530 at a dose of 50 mg/kg also significantly inhibited the delayed type hypersensitivity (DTH) response to type II collagen, but not the production of anti-type II collagen IgG antibody in arthritic mice.

To investigate the inhibitory mechanism of TRK-530, the type of effect of TRK-530 on the production of IL-1 β *in vitro* was studied. TRK-530 at a concentration of 10^{-4} M inhibited LPS-induced IL-1 β production from J774.1 cells.

In conclusion, TRK-530 inhibited CIA in mice. The inhibition of the DTH reaction to type II collagen and the inhibition of IL-1 β production may partly participate the anti-rheumatoid action of TRK-530.

Key words TRK-530; collagen-induced arthritis; interleukin-1 β (IL-1 β)

TRK-530 is a derivative of diphosphonates (DPs). As DPs have high affinity to bone^{1,2)} and a suppressive effect on the excessive resorption of bone,³⁾ they are used to treat mineralization disorders associated with Paget's disease,⁴⁾ multiple myeloma of bone⁵⁾ and osteoporosis.^{6,7)} Recently, Dunn *et al.* reported trials involving the experimental therapy of rheumatoid arthritis (RA) with DPs in animals.^{8–10)}

RA is well known to be characterized by progression and chronic non-specific inflammation with various immune abnormalities. Histopathological studies of RA synovium demonstrated the marked proliferation of synovial cells and the infiltration of lymphocytes in association with the destruction of cartilage and bone. Moreover, many investigators pointed out that the destruction of joint bone is related to the production of oxygen radicals^{11,12)} and of various mediators including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α)^{13–15)} and IL-6^{16–18)} by macrophage-like cells.

Several previous investigations^{1,2,19,20)} have indicated that DPs show beneficial pharmacological profiles including anti-oxidant action, Ca²⁺ binding action and the ability to induce the apoptosis of osteoclast cells for the therapy of RA. The present studies have, therefore, been conducted to investigate the effect of TRK-530, a DP derivative, on collagen-induced arthritis (CIA) in mice.

MATERIALS AND METHODS

Animals Male DBA/1J mice weighing 20–30 g were used. Animals were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in plastic cages in an air-conditioned room at 24 °C, fed a standard laboratory diet, and given water *ad libitum*.

Drugs Disodium dihydrogen [4-(methylthio)phenylthio]methanediphosphonate (TRK-530) was donated by Toray Industries, Inc. (Tokyo, Japan). Prednisolone ace-

tate (Pred; Shionogi Co., Ltd., Osaka, Japan) and indomethacin (Ind; Sigma Chemical Co., MO, U.S.A.) were purchased commercially. Each drug was administered by subcutaneous injection.

CIA in Mice CIA was induced in DBA/1J mice according to the method previously described.²¹⁾ Seven-week-old male DBA/1J mice were injected intradermally at the back of the tail with 200 μ g of bovine type II collagen (Cosmobio, Tokyo, Japan), suspended in 0.01 mol/l acetic acid and emulsified in complete Freund's adjuvant (Nacalai Tesque, Inc., Kyoto, Japan). Three weeks after the initial injection, a booster injection of 200 μ g of bovine type II collagen emulsified in complete Freund's adjuvant was injected intradermally at the base of the tail.

Evaluation of clinical arthritis activity was carried out at 0, 3, 4, 5, 6, 7, 8 and 9 weeks after the initial immunization. The degree of arthritis in the extremities of these animals was evaluated clinically by the same observer. The severity of arthritis in the metacarpophalangeal wrist, metatarsophalangeal and ankle joints was scored as 0=no arthritis, 1=small degree of arthritis, 2=light swelling, 3=medium swelling, 4=severe swelling, 5=severe swelling and non-weight-bearing. The joint score was the sum of the scores of all involved joints. The arthritis index was calculated by averaging the total scores. In addition to the arthritis index, the change in paw volume as measured by plethysmometer (TK-101; Unicom, Chiba, Japan) was used to determine the severity of arthritis. At the end of the experimental period (9 weeks), radiographic assessment of skeletal changes was performed using a

Fig. 1. Chemical Structure of TRK-530

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Softex X-ray mechanism (Shimazu, Kyoto, Japan). Bone changes were graded on a scale of 0–2; 0=negative, 1=subtle small erosions, 2=obvious large erosions in multiple joints. The radiographic score was the sum of the scores of the four legs. The animals were killed and the limbs from positive and negative control groups were then amputated and immersed in 10% neutral formalin. The joints were decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histopathological analysis was carried out, observing degeneration of cartilage, the formation of pannus and the infiltration of cells was observed. The severity of the lesions was classified into four grades; – =negative; + =slight; ++ =mild; +++ =marked.

Measurement of the Humoral and Cellular Response in CIA Mice At the end of the test, the cell mediated immune response of the mice to collagen was estimated as follows; The thickness of both ears of each mouse was measured using a dial thickness gauge (R2-1A, Ozaki Co., Ltd., Tokyo, Japan). Ten micrograms of type II collagen in phosphate buffered saline, pH 7.0, was injected into each ear, and the ear thickness of each animal as noted at 24h was used as the measurement of *in vivo* cell-mediated immunity (delayed type hypersensitivity; DTH) to collagen. After termination of the experiment, all mice were bled by puncture of the ophthalmic venous plexus and the sera were analyzed at a dilution of 1:100000 for IgG anti-type II collagen antibody levels using the ELISA technique described by Phadke *et al.*²²⁾

The Production of IL-1 β from J774.1 Cell Murine derived macrophage-like cell, J774.1, was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μ M mercaptoethanol, 50 U/ml penicillin, and 50 μ M streptomycin (FCS-RPMI). The cells were adjusted to a concentration of 2×10^5 cells/ml using FCS-RPMI and plated into 24-well plates (Corning Coster Co., MA., U.S.A.) at 1 ml/well. After incubation at 37 °C for 30 min in 5% CO₂, lipopolysaccharide (LPS; Difco Laboratories, MI, U.S.A.) was added to a final concentration of 1 μ g/ml. After incubation for 24 h, the supernatant was harvested. The supernatant was centrifuged to remove cell debris and

then IL-1 β was assayed by ELISA (Endogen, Inc., MA, U.S.A.).

Statistics Results were statistically evaluated by Dunnet's multi comparison method. Evaluation of the arthritis index and the radiographic score were done using the Mann-Whitney method.

RESULTS

Effect on CIA The immunization of DBA/1J mice with bovine type II collagen resulted in polyarthritis in all animals after secondary immunization. After secondary immunization, at which time the redness and edema of the footpad and the development of arthritis were observed, TRK-530 at doses of 25 and 50 mg/kg, Pred at a dose of 5 mg/kg and Ind at a dose of 2 mg/kg inhibited the development of arthritis (Fig. 2). In a histopathological study, chronic proliferative synovitis, including the degeneration and erosion of cartilage and the formation of pannus in knee regions, was observed in the control group. TRK-530 significantly prevented the degeneration of cartilage, the formation of pannus and infiltration of cells at a dose of 50 mg/kg. Pred also inhibited the degeneration of cartilage, pannus formation and infiltration of cells to the synovial layer (Table 1). In evaluating bone destruction using soft-X-ray photography, the injection of type II collagen produced the bone destruction in the knee and tarsal joints. TRK-530 at a dose of 50 mg/kg inhibited this bone destruction (Fig. 3).

Effect on Immune Response in CIA Mice In arthritic mice, the anti-type II collagen antibody level reached its peak 5 weeks after the primary immunization (data not shown). Pred inhibited the production of antibody to type II collagen. TRK-530 at a dose of 50 mg/kg and Ind showed a tendency to inhibit the production of antibody. On the other hand, the DTH to type II collagen in arthritic mice at 9 weeks after the primary immunization was clearly inhibited by treatment with TRK-530 (50 mg/kg), Pred and Ind (Table 2).

Effect of IL-1 β Production from J774.1 Cells After stimulation with LPS, 275 pg/ml IL-1 β was released from

Fig. 2. Effect of TRK-530, Pred and Ind on CIA in DBA/1J Mice for Arthritis Index [A] and Footpad Volume [B].

TRK-530, Pred and Ind were administered s.c. once a day for 9 weeks. Each group consists of 7 animals. *, ** Statistically significant from vehicle at $p < 0.05$ and $p < 0.01$, respectively.

Table 1. Effect of TRK-530, Pred and Ind on Type II Collagen-Induced Arthritis in DBA/1J Mice for Histopathological Changes

Organ finding	Grade	Vehicle	TRK-530 (mg/kg)			Pred (5 mg/kg)	Ind (2 mg/kg)
			10	25	50		
Degeneration of cartilage	—	0	0	4	7	14	3
	+	2	11	8	7	0	6
	++	11	3	2**	0**	0**	5
	+++	1	0	0	0	0	0
Formation of pannus	—	1	0	9	10	14	9
	+	4	10	5	3	0	5
	++	8	3	0**	1**	0**	0**
	+++	1	1	0	0	0	0
Infiltration of cells	—	0	0	0	6	14	3
	+	5	8	12	6	0	5
	++	8	5	2	2**	0**	6
	+++	1	1	0	0	0	0

The drugs were administered s.c. at various doses 6 times/week for 9 weeks. Grade: — = negative; + = slight; ++ = mild, +++ = marked. ** $p < 0.01$ vs. vehicle.

Fig. 3. Effect of TRK-530, Pred and Ind on CIA in DBA/1J Mice for Radiographic Score.

TRK-530, Pred and Ind were administered s.c. once a day for 9 weeks. Each group consists of 7 animals. *, ** Statistically significant from vehicle at $p < 0.05$ and $p < 0.01$, respectively.

J774.1 cells, compared to 31 pg/ml without LPS. TRK-530 (10^{-4} M), Pred (10^{-5} M) and Ind (10^{-5} M) decreased the release of IL-1 β to 116, 32 and 178 pg/ml, respectively (Fig. 4). The viability of J774.1 cells was not affected by TRK-530, Pred or Ind at those doses (data not shown).

DISCUSSION

The present study evaluated the anti-arthritis activity of TRK-530 using CIA in mice at three different doses (10, 25 and 50 mg/kg) with reference to that of Pred (5 mg/kg) and Ind (2 mg/kg). TRK-530 at a dose of 50 mg/kg inhibited the progression of arthritis with regard to the disease's clinical (edema and bone destruction) and histopathologic (degeneration of cartilage, formation of pannus and infiltration of cells) manifestations. TRK-530 at a dose of 25 mg/kg also showed slight inhibitory action on the progression of the arthritis index and footpad swelling. This suppressive potency with TRK-530 (50 mg/kg) was almost the same as that of Pred or Ind.

To investigate the inhibitory mechanism of TRK-530, its effects on humoral and cell-mediated immunity to type II collagen in arthritic mice were studied. Many previous reports indicated the participation of T cells in the development of CIA.^{23,24} TRK-530 and Ind suppressed the DTH reaction to type II collagen in a dose related

Table 2. Effect of TRK-530, Pred and Ind on Collagen-Induced DTH and Anti-Type II Collagen Antibody in DBA/1J Mice

Drug	Dose (mg/kg)	N	Swelling ($\times 10^{-3}$ cm)	Anti-type II collagen antibody (OD)
Vehicle		7	26.4 $\pm$ 2.30	0.817 $\pm$ 0.132
TRK-530	10	7	23.5 $\pm$ 2.38	0.599 $\pm$ 0.164
	25	7	22.9 $\pm$ 2.10	0.502 $\pm$ 0.138
	50	7	19.6 $\pm$ 3.66*	0.567 $\pm$ 0.164
Pred	5	7	12.9 $\pm$ 0.99**	0.201 $\pm$ 0.120**
Ind	2	7	15.5 $\pm$ 1.29**	0.468 $\pm$ 0.054

The drugs were administered s.c. 6 times/week for 9 weeks. Collagen-induced DTH was carried out 9 weeks after the primary immunization. Ear swelling was measured 24 h after the challenge. Anti-type II collagen antibody was measured in mice 5 weeks after the primary immunization. * $p < 0.05$, ** $p < 0.01$ vs. vehicle. OD = optical density.

Fig. 4. Effect of TRK-530, Pred and Ind on LPS-Induced IL-1 β Production.

J774.1 cells were stimulated with or without 1 μ g/ml LPS for 24 h. The results are expressed as mean $\pm$ SE (N=4). ** Statistically significant from the no drug group at $p < 0.01$.

fashion, but did not suppress the anti-type II collagen antibody. Pred suppressed both reactions. There are many reports which suggest the participation of T cells, which produce interferon (IFN- γ)^{25,26} and express the mRNA of IFN- γ ,^{27,28} especially in the connective tissue of joints, in the onset and development of RA. This evidence suggests an important role of T helper 1 (Th1) in the onset of RA. The DTH reaction is mainly mediated by Th1 cells. The present results, therefore, suggest that the inhibition of the DTH reaction to type II collagen is related to the suppression of CIA in mice. The above mechanism is clearly demonstrated at a dose of 50 mg/kg TRK-530, but not at a dose of 25 mg/kg. Direct inhibition of footpad swelling may be another inhibitory mechanism of TRK-530. Further experiments will be necessary to clarify the direct anti-inflammatory action of TRK-530.

In addition, TRK-530 inhibited the LPS-induced IL-1 β production from a macrophage-like cell, J774.1. Some investigators reported that an inflammatory cytokine, especially IL-1 β , is involved in the onset and development of RA and experimental arthritis. In experimental arthritis, a monoclonal antibody against IL-1 β ²⁹⁻³² and IL-1 receptor antagonist³²⁻³⁵ suppressed the onset and development of arthritis. Moreover, many clinical studies indicate the role of IL-1 β in the onset and development of arthritis.³⁶⁻³⁹ Among them, Lent *et al.* reported that the treatment of diphosphonate induced the inhibition of IL-1 β production and then the suppression of CIA. They have suggested that phagocytic lining cells play a crucial role in the local expression of inflammation in systemically induced CIA, and that IL-1 β probably activated phagocytic lining cells.⁴⁰ TRK-530 may inhibit the activation of the phagocytic lining cell. However TRK-530 at only an relatively high concentration, such as 10^{-4} M, decreased the production of IL-1 β from the murine macrophage-like cell, J774.1. Therefore, this IL-1 β inhibition may only partially contribute to the failure to induce CIA. On the other hand, Ind had no effect on LPS-induced IL-1 β production.

In conclusion, TRK-530, a derivative of DPs, showed a suppressive effect on the T cell mediated DTH reaction. The inhibition of IL-1 production may partially participate the inhibition of CIA. These results suggest that TRK-530 has a different inhibitory mechanism on CIA than those of Pred and Ind; therefore, TRK-530 may be promising for the treatment of RA with few side effects.

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