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Tranilast Suppresses the Disease Development of the Adjuvant- and Streptococcal Cell Wall-Induced Arthritis in Rats

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Abstract. This study explores the effects of the anti-allergic and anti-fibrotic agent tranilast on adjuvant- and streptococcal cell wall-induced arthritis in rats, animal models of rheumatoid arthritis in humans. Tranilast (150 or 300 mg/kg, twice daily) or vehicle only was administered orally to the two arthritis models, from 17 days before sensitization. As a comparative control, methotrexate (0.1 mg/kg, once daily) was given to another group. Tranilast suppressed the increase in foot volumes, paw thicknesses, clinical scores, and histopathological scores of the ankle joints in both models dose-dependently. In addition, the fibrosis indices of the ankles were dramatically decreased by tranilast in both of the models. Compared to the effects of methotrexate, tranilast seemed to work more effectively in the streptococcal cell wall-induced arthritis model than in the adjuvant-induced arthritis model. From these observations, it can be concluded that tranilast suppresses the development of arthritis in multiple models and is potentially a novel therapeutic agent for human rheumatoid arthritis.

Keywords: tranilast, rheumatoid arthritis, adjuvant-induced arthritis, streptococcal cell wall-induced arthritis, methotrexate

Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovial joints, which leads to progressive destruction of the cartilage and the bone (1). Work disability is seen in 60% of RA patients after 10 years of the disease, and mortality is increased in patients with severe RA (2). Among the animal RA models, adjuvant-induced arthritis (AIA) and streptococcal cell wall-induced polyarthritis (SIA) have made large contributions to the development of therapeutic drugs (3, 4). In fact, AIA and SIA have been extensively studied since the 1950's and are the most widely used arthritic rat models for the purpose of predicting the outcome of drug treatments (5 – 8). Of these, methotrexate (MTX), the "gold standard" disease-modifying anti-rheumatic drug (DMARD) used to treat human RA, is also known to be effective in both models when the drug is administered prophylactically (3, 8, 9).

Tranilast, or *N*-(3',4'-dimethoxycinnamoyl) anthranilic acid, is a synthetic, multi-potential, anti-allergic, and anti-fibrotic drug reported to have various effects both in vitro as well as in vivo (10). The variety of tranilast functions published to date include suppression of collagen synthesis by fibroblasts via down-regulation of cytokine release from monocytes/macrophages (11), transcriptional and translational inhibition of matrix metallo-proteases in lipopolysaccharide-stimulated neutrophils (12), and suppression of monocyte/macrophage infiltration and associated myocardial fibrosis in the deoxycorticosterone acetate/salt hypertensive rat (13). Recently, Platten and colleagues have reported that tranilast, as a metabolite of tryptophan, reverses the paralysis in experimental autoimmune encephalomyelitis, which is an established model for multiple sclerosis (MS) in humans (14). They described that tranilast altered the cytokine release by lymphocytes both in vivo and in vitro, which suggested the possibility that it could cure not only MS, but also the majority of autoimmune disorders including RA.

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The present study was done to elucidate, if any, the effects of tranilast in two RA models, AIA and SIA, and to explore for the possibility of tranilast becoming a therapeutic agent for human RA. Additionally, we aimed to compare tranilast with existing remedies, so effects of MTX on AIA and SIA were evaluated at the same time.

Materials and Methods

Animals

Lewis rats (LEW/*CrlCrlj*), which were specific pathogen-free, were purchased from Charles River Japan Inc. (Osaka). After a 7-day quarantine/acclimatization-period, the animals were separated into groups by a repeated stratified randomization using the latest measured body weights. The animals were housed in individual cages and had free access to water and food throughout the study. All animal in-life procedures were approved by the Institutional Animal Care and Use Committee of the Toxicology Research Laboratories of Kissei Pharmaceuticals Co., Ltd.

AIA study

Mycobacterium butyricum desiccated (Difco Laboratories, MI, USA) was thoroughly ground and squalene (Nacalai Tesque, Inc., Osaka) was gradually added to 7 mg/ml. The suspension was then incubated overnight at 100°C. Ten-week-old male rats were anesthetized with isoflurane and injected intradermally with the suspension at two sites on the dorsal side of their tail root, at 0.05 ml per site, for a total volume of 0.1 ml. The study consisted of five groups: Intact (non-sensitized) (n = 5); Vehicle (n = 10); Tranilast, 150 mg/kg (n = 10); Tranilast, 300 mg/kg (n = 10); and MTX, 0.1 mg/kg (n = 5). Additionally, five more animals per group were recruited for spleen sampling.

SIA study

PG-PS 10S (Lee Laboratories, GA, USA) solution was sonicated by a probe-type sonicator immediately before application and injected intraperitoneally into 9-week-old female rats to a total of 15 μg of rhamnose /gram average body weight. The study consisted of five groups: Intact (non-sensitized) (n = 5); Vehicle (n = 10); Tranilast, 150 mg/kg (n = 10); Tranilast, 300 mg/kg (n = 10 until Day 12 and n = 9 from Day 14, due to one dropout on Day 13 post-sensitization); MTX, 0.1 mg/kg (n = 5).

Drugs

Tranilast or MTX (Wako Pure Chemical Industries, Ltd., Osaka) was suspended in 0.5% carboxymethyl cellulose (CMC) solution and administered by oral gavage. Intact and vehicle animals were given 0.5% CMC only. Animals were dosed twice daily, except for MTX rats, which received treatment once daily. All animals started their treatment 17 days prior to sensitization.

Clinical parameters

All parameters were measured in a blinded manner to avoid data bias. Foot volumes of the animals were measured by volume meter Model TK-105 (Muromachi Kikai Co., Ltd., Tokyo). Paw thicknesses were measured by a slide caliper. Clinical scores of the limbs were recorded in accordance to criteria described previously (15). In brief, each limb was scored according to a 5-point scoring system (0: no swelling or erythema, 1: slight swelling and/or erythema, 2: low to moderate edema, 3: pronounced edema with limited joint usage, and 4: excess edema with joint rigidity); and the sum was considered as the clinical score, ranging from 0 to 16.

Histopathological investigations

The left hind limbs, including the knee and tarsal joints, were fixed and preserved in 10% phosphate-buffered formalin. After decalcification, the tissues were embedded in paraffin, sectioned at 5-μm thickness and stained with hematoxylin and eosin (H&E). The H&E preparations were microscopically observed focusing on the knee joint, the distal tibia-talocrural joint, the calcaneus-subtalar joint, and the tarsus. The scoring methods were based on previous reports (16, 17). In brief, the sum of the 4-point scoring system (0: no significant changes, 1: mild, 2: moderate, 3: marked) was considered to be the histopathological score of a section, and the average score of each group was compared for bone erosion, new bone formation, pannus formation, cartilage destruction, synovial cell hyperplasia, inflammatory cell infiltration, circumarticular fibrosis, and edema. Serial talocrural joint sections were also stained by Azan's method, and the percentage of the aniline-blue positive areas was measured by a LUZEX 3 computer-assisted image analyzer (Nikon, Tokyo) and was considered as an individual fibrosis index.

Statistics

All values are expressed as means ± S.D. Statistical analyses were carried out using one-way analysis of variance followed by Sheffe's Method.

Results

Tranilast suppresses disease development in AIA and SIA models

In the two rat models, tranilast was found to suppress the development of the signs of the clinical disease. In the AIA study (Fig. 1), foot volumes, paw thicknesses, and clinical scores of the vehicle-treated animals began to increase from Day 8 post-sensitization and peaked on Day 18, followed by a slight attenuation. Tranilast suppressed the increase of these parameters in a dose-dependent manner, which became statistically significant at the dose of 300 mg/kg compared with the vehicle from Day 12 onwards. MTX completely suppressed the increase of these parameters after Day 10. In the SIA study (Fig. 2), the animals treated with vehicle only showed typical biphasic inflammatory responses in foot volumes, paw thicknesses, and clinical scores; the acute phase reaction peaked on Day 2, was followed by a temporary remission, and then aggressive and consecutive chronic phase reactivation appeared. The animals treated with tranilast showed dose-dependent reductions in the three parameters in both acute and chronic phases and the reductions were significant even at the lower (150 mg/kg) dosage level. Unlike the AIA study, MTX-treated animals showed a much different disease course,

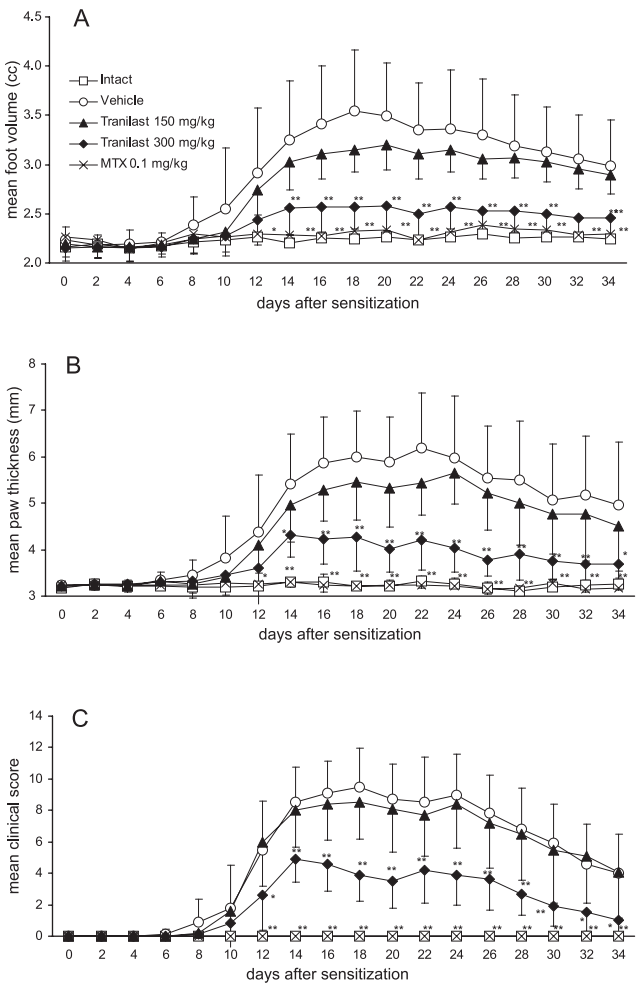

Fig. 1. Effects of tranilast and MTX on the prevention of disease development in AIA model rats. Foot volumes (A), paw thicknesses (B), and clinical scores (C) were measured every second day following sensitization. Intact (n = 5) and Vehicle (n = 10) animals were given 0.5% CMC twice daily, as was tranilast at doses of 150 (n = 10) or 300 mg/kg (n = 10). MTX at 0.1 mg/kg was administered once daily. For each item, Tranilast and MTX groups were compared statistically with the vehicle group (**P*<0.05, ***P*<0.01).

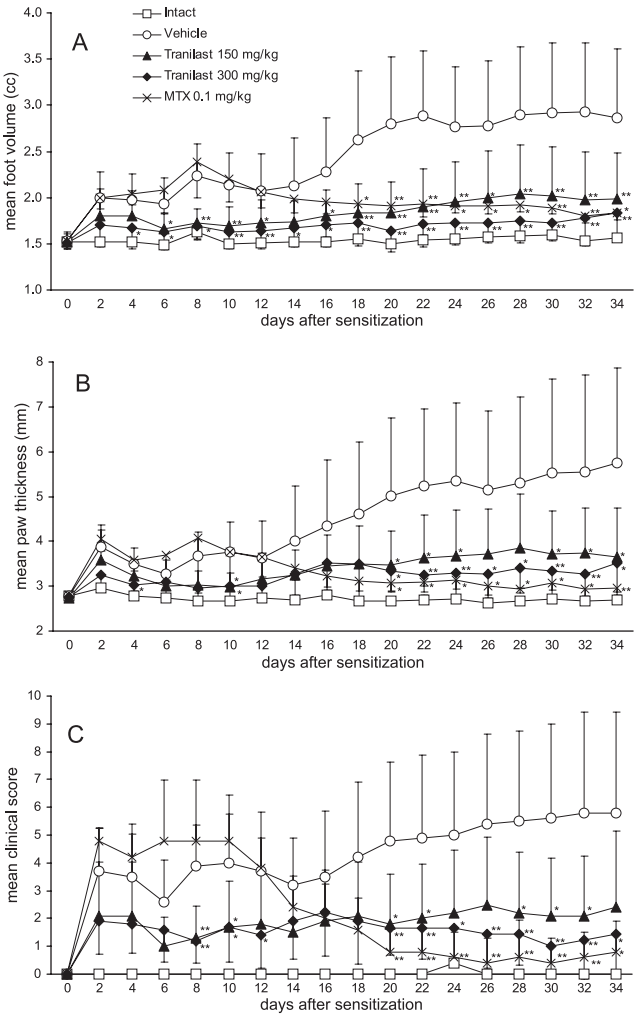

Fig. 2. Effects of tranilast and MTX on the prevention of disease development in SIA model rats. Foot volumes (A), paw thicknesses (B), and clinical scores (C) were measured on every second day following sensitization. Intact (n = 5) and Vehicle (n = 10) animals were given 0.5% CMC twice daily, as was tranilast at 150 (n = 10) or 300 mg/kg (n = 10 until Day 12 and n = 9 from Day 14 due to one dropout on Day 13). MTX at 0.1 mg/kg was administered once daily. For each item, Tranilast and MTX groups were compared statistically with the vehicle group (**P*<0.05, ***P*<0.01).

with a single peak on Day 6, followed by a decrease in foot volumes, paw thicknesses, and clinical scores. The reduction in the three parameters in the MTX-treated animals became significant on Day 18, although its effect never exceeded that of 300 mg/kg tranilast.

Tranilast reduces the histopathological scores and fibrosis indices in AIA and SIA models

For a more precise understanding of the effect of tranilast, arthritic lesions were microscopically investigated and scored. In the AIA study (Table 1A, Fig. 3), bone erosion in the medullary portion, new bone formation, pannus formation, articular cartilage destruction, stratification or cystic hyperplasia of synovial cells, inflammatory cell infiltration, circumarticular fibrosis, and edema were found in the animals treated with vehicle only. Animals administered 300 mg/kg of tranilast showed a reduction in all of the items compared with vehicle-treated rats, while those treated with MTX at 0.1 mg/kg showed virtually no adverse differences from the intact animals. In the SIA study (Table 1B, Fig. 4), vehicle-treated rats reacted similarly to those in the AIA study. However, in this model, a noticeable decrease in the parameters was found even in the 150 mg/kg tranilast group. These parameters were suppressed further in the 300 mg/kg tranilast group, and the animals dosed with MTX at 0.1 mg/kg also showed a strong reduction in the findings.

Additionally, the fibrosis index of the left pericalneous connective tissue was measured in each AIA and SIA animal (Fig. 5). Tranilast dose-dependently reduced the animals' fibrosis indices and this reduction was significant in both models. MTX-dosed animals also showed reduced fibrosis indices.

Discussion

In the present study, we evaluated the anti-allergic and anti-fibrotic agent tranilast with regards to its applicability to treat RA. The concept that tranilast might be effective against RA originated from an earlier report that tranilast inhibited proliferation, vascular endothelium growth factor (VEGF)-induced chemotaxis, and in vitro angiogenesis of human dermal microvascular endothelial cells and in vivo angiogenesis in mice (18). The importance of angiogenesis and chemotaxis in RA is discussed elsewhere (19 – 21). In addition, there is a recent report that tranilast, other than anti-angiogenic or anti-VEGF-induced chemotactic effects, had suppressive effects in autoimmune disease models (14). Taken together, these previous reports led us to the idea of testing tranilast on RA models.

The two rat models selected as test systems, AIA and SIA, have been extensively used for the evaluation of

Table 1. Histopathologic scoring of animals in the AIA study (A) and SIA study (B)

Histopathological findings	Intact	Vehicle	Tranilast (150 mg/kg)	Tranilast (300 mg/kg)	MTX (0.1 mg/kg)
A. AIA					
Bone erosion	0	1.50 ± 2.22	1.30 ± 2.11	0.33 ± 0.71	0
New bone formation	0	3.20 ± 2.39	2.90 ± 1.91	1.22 ± 1.39	0
Pannus formation	0	1.20 ± 1.23	0.70 ± 1.06	0.44 ± 0.73	0
Cartilage destruction	0	0.70 ± 1.34	0.50 ± 0.85	0	0.20 ± 0.45
Synovial cell hyperplasia	0	2.10 ± 1.45	2.30 ± 1.25	2.00 ± 1.94	0
Inflammatory cell infiltration	0	2.40 ± 1.43	2.80 ± 1.14	1.56 ± 1.51	0.20 ± 0.45
Circumarticular fibrosis	0	4.10 ± 2.33	3.50 ± 2.42	1.67 ± 1.80	0
Edema	0	1.20 ± 1.48	1.60 ± 2.37	0.22 ± 0.67	0
B. SIA					
Bone erosion	0	5.30 ± 2.91	2.00 ± 2.87	1.00 ± 1.32	0
New bone formation	0	6.30 ± 3.47	2.10 ± 2.23	0.78 ± 0.67	0.60 ± 0.89
Pannus formation	0	3.00 ± 2.40	1.60 ± 1.96	1.00 ± 1.00	0
Cartilage destruction	0	5.30 ± 2.95	1.10 ± 1.45	0.44 ± 0.73	0
Synovial cell hyperplasia	0	4.20 ± 2.66	2.30 ± 2.36	3.22 ± 2.68	1.80 ± 1.10
Inflammatory cell infiltration	0	3.20 ± 1.81	1.70 ± 1.70	3.00 ± 2.50	2.00 ± 1.41
Circumarticular fibrosis	0	5.60 ± 2.59	2.90 ± 2.88	2.22 ± 2.64	2.40 ± 1.14
Edema	0	4.90 ± 3.14	1.20 ± 2.30	0.78 ± 1.99	0

Each value is a mean ± S.D.

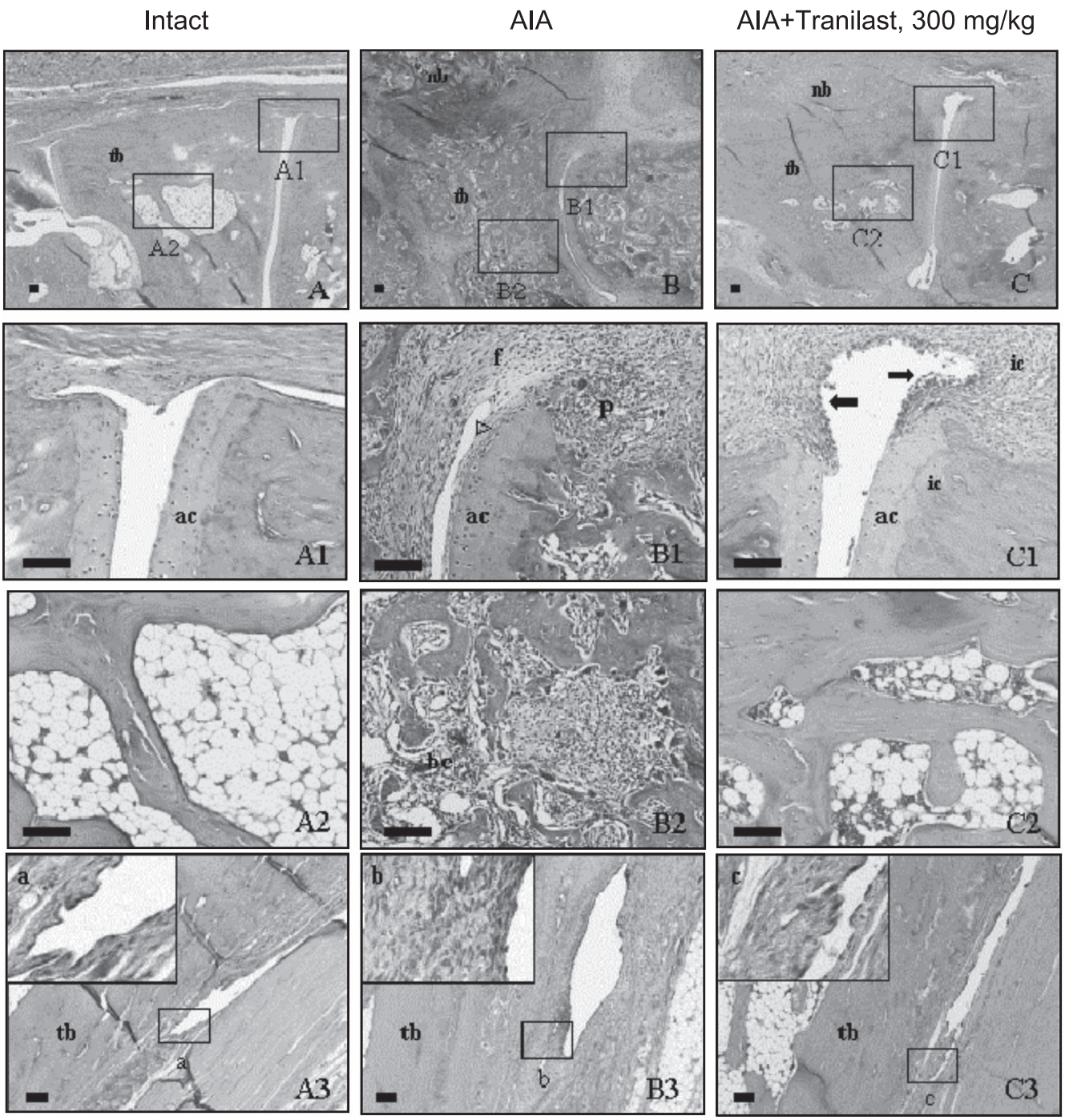

Fig. 3. Histopathological findings of tarsal joints (Photo A: intact; B: AIA; C: AIA + Tranilast, 300 mg/kg) and the synovial membrane of tibia (Photo A3: intact; B3: AIA; C3: AIA + Tranilast, 300 mg/kg, insets are ×5 magnification) in the AIA study. H&E stain. Each scale bar is 100 μm. No abnormal findings were observed in an intact rat (Photos A, A1, A2, and A3). Photo B: Marked new bone formation (nb) on the tarsal bones (tb) was observed. Photo C: Modeate new bone formation was observed. Photo B1: Circumarticular fibrosis (f), pannus formation (p), and cartilage destruction (open arrowhead) were observed. Photo C1: Slight synovial cell hyperplasia (closed arrows) and inflammatory cell infiltration (ic) were observed, but no evident pannus formation and cartilage destruction. Photo B2: Marked bone erosion (be) was observed. Photo C2: No abnormalities were observed. Photo B3: Marked synovial cell hyperplasia (b) and fibrosis (f) were observed. Photo C3: Note that synovial hyperplasia (c) and fibrosis were slight.

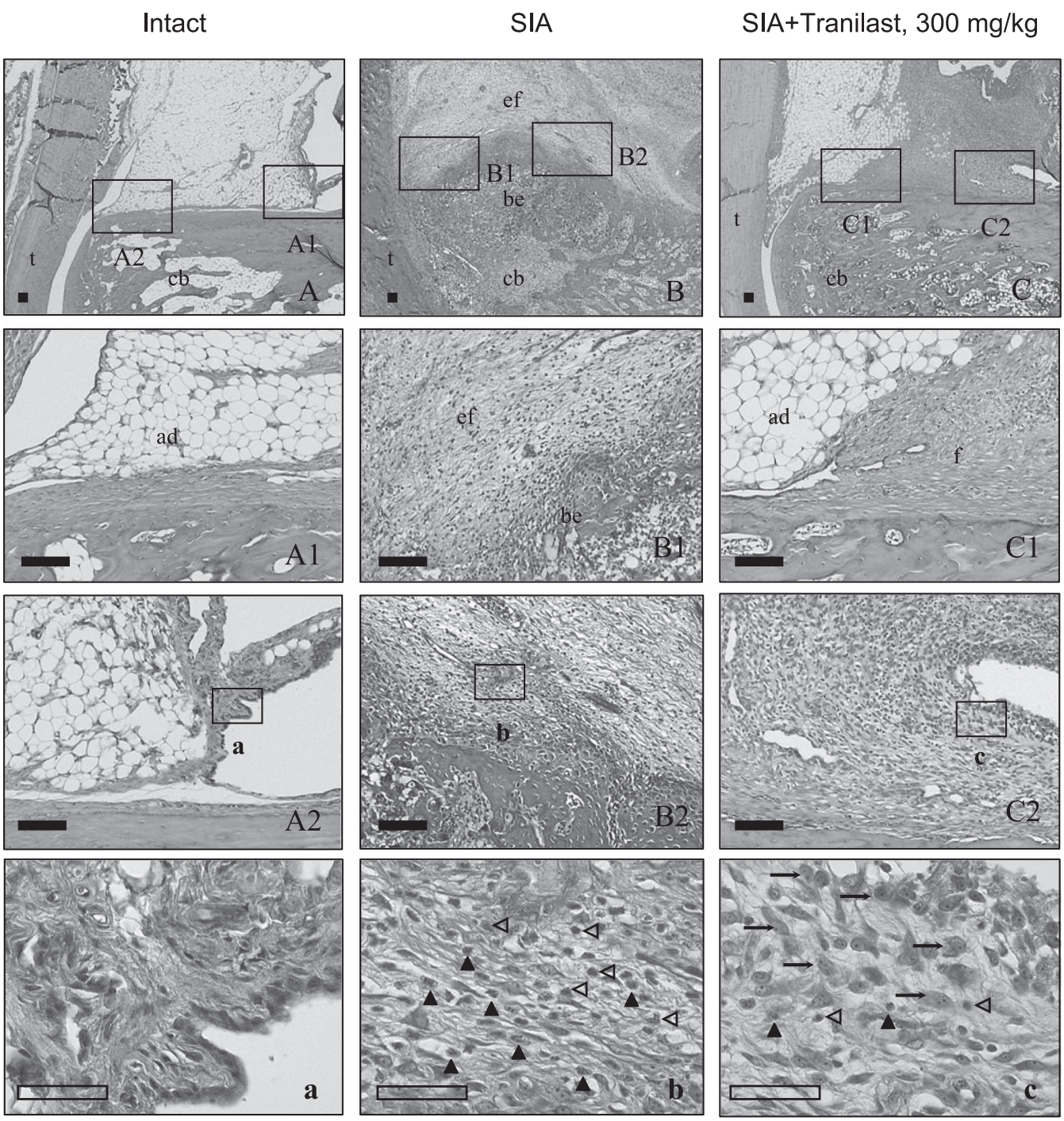

Fig. 4. Histopathological findings of calcaneus bone (cb) and its circumarticular tissue (Photo A: intact; B: SIA; C: SIA + tranilast, 300 mg/kg) in the SIA study. H&E stain. Closed scale bars and open scale bars are 100 and 50 mm, respectively. No abnormal findings were observed in an intact rat (Photos A, A1, A2, and A3; t: tendon). Photo B: Marked bone erosion (be) and edematous fibrosis (ef) were observed. Cartilage of calcaneus bone was almost completely destroyed. Photo C: Moderate fibrosis (f) was observed. The cartilage and bone showed normal appearance. Photo B1: Edematous fibrosis (ef) with concomitant decrease in adipocytes was observed. Photo C1: Note that fibrosis with concomitant decrease in adipocytes (ad) was moderate. Photo B2&b: Marked inflammatory cell infiltration consisting of a number of neutrophils (closed arrowheads) and macrophages (open arrowheads) and their debris was observed. Photo C2&c: Note that inflammatory cell infiltration was moderated and the number of neutrophils (closed arrowheads) and macrophages (open arrowheads) were decreased, while synovial cell hyperplasia (arrows) was prominent.

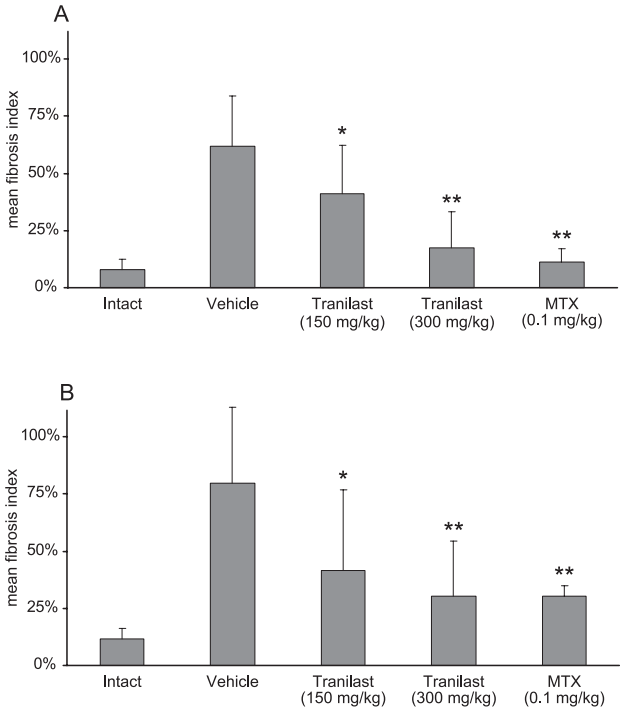

Fig. 5. Effects of tranilast and MTX on fibrosis index in terminal histopathologic investigations. Animals of the Intact (n = 5); Vehicle (n = 10); Tranilast, 150 mg/kg (n = 10); Tranilast, 300 mg/kg (n = 10 for AIA, n = 9 for SIA); and MTX, 0.1 mg/kg (n = 10) groups received their regimen for 34 days and were then euthanized. For the fibrosis index, tranilast- and MTX-administered animals from the AIA study (A) and SIA study (B) were compared statistically with vehicle treated animals (**P*<0.05, ***P*<0.01).

RA drugs and showed the same reactions as found in literature (3). The similarities and differences between AIA and SIA are well known. In T cells, the antigenic cross-reactivity between components of the streptococcal cell wall and mycobacterial antigens have been reported (22); whereas the AIA disease process is monophasic and eventually remits, SIA is biphasic and persistent. In addition, the AIA process is entirely T cell-dependent, while in the SIA model, the acute phase is T cell-independent (23). Having this difference between the two models built-in to the experimental design made it presumable that this study could elucidate, at least partially, the mechanism of tranilast.

MTX was chosen as a control agent to compare the efficacy of tranilast to standard therapies, to gauge if the test system was operating correctly, and to offer insight into the modes of action of tranilast. MTX is often chosen as a control drug in the clinical development of anti-rheumatics (24), and it is known to affect the cytokine gene expression in lymphocytes towards Th2 from Th1, while at the same time reducing the production of proinflammatory monocytic/macrophagic cytokines (25).

Table 2. Table of the differences in the modes of actions between MTX and tranilast

	MTX	Tranilast	T cell dependence
SIA			
acute	–	+	–
chronic	+	+	+
AIA	++	+	+

In both of the RA models, we were able to show that tranilast prevented the disease development in a dose-dependent fashion in clinical and histopathological tests. The increase in the foot volumes, paw thicknesses and clinical scores observed after sensitization were dramatically suppressed by treatment with tranilast. Additionally, the terminal histopathological scores and fibrosis indices were reduced in the tranilast-treated animals in both models. MTX suppressed the disease in both models, consistent with other reports (8, 9). What was so interesting was that in the acute phase of the SIA model, tranilast was measured to exceed the performance of MTX. This observation presumably lies in the differences between the pathologies of the two models; the acute phase reaction of SIA is a complement-dependent T cell-independent pathology, whereas T cells are essential for the reactivation in the chronic phase (22). Thus, it is likely that T cell-dependent phases are susceptible to MTX and MTX's major cellular target is T cells (22). On the other hand, tranilast suppressed both of the phases, which suggests that the main cellular target of tranilast is at least, but not limited to, T cells (Table 2).

The purpose of the 17-day pretreatment in the present study was to optimize the efficacy of tranilast. Previous reports have shown that in the clinical usage of tranilast, pre-treatment with the drug resulted in enhanced preventive effects against pollinosis and hypertrophic scarring (26, 27). Additionally, MTX is also suggested to work more effectively when it is given in a prophylactic regimen (9, 28), although there is still the question of whether this 17-day period can be optimized to show the best performance of tranilast in the two models, and further attempts are being planned.

It is already well known that tranilast has an inhibitory effect on the development of fibrosis (29, 30), and in the two RA models in the present study, tranilast again showed its ability as an anti-fibrotic agent. However, the mechanism for this effect in these models remains unclear, especially with regards to the anti-inflammatory effects of tranilast. Since the fibrosis indices in both of

the models were significantly reduced by tranilast, it is possible that tranilast may selectively suppress fibrosis more than other events in the inflammation pathway related to arthritis.

In conclusion, we have shown that tranilast suppresses the development of disease in both AIA and SIA models of human RA, presumably in a T cell-independent manner. Although treatment outcomes varied from MTX, the pathology of RA is of great diversity and cannot be wholly explained by a single model (3). As such, our results that tranilast was effective on multiple animal models and in a different mode of action from MTX supports the possibility that this agent may be a promising candidate for the treatment of human RA, at least in a therapeutic combination with MTX. Future studies are currently being planned to examine the ideal doses and potential combination treatment modalities of tranilast and other RA drugs such as MTX.

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