British Journal of Pharmacology (2007) 150, 862–872
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents

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Background and purpose: Rheumatoid arthritis (RA) is a chronic inflammatory disease. Histone deacetylase inhibitors (HDACi), a new class of anti-cancer agents, have recently been reported to exhibit potent anti-inflammatory activities. A proof of concept study was carried out with suberoylanilide hydroxamic acid (SAHA) and MS-275, two HDACi currently undergoing clinical investigations for various oncological indications.
Experimental approach: The anti-rheumatic effects of SAHA and MS-275 were assessed in both mouse and rat collagen induced arthritis (CIA) models.
Key results: SAHA exhibited moderate prophylactic efficacy. It attenuated paw swelling due to inflammation, decreased bone erosion in both mice and rats and reduced slightly the RA-induced bone resorption in rats. However, SAHA could not inhibit the onset of arthritis. In contrast, MS-275 displayed dramatic anti-rheumatic activities. In prophylactic intervention, high doses of MS-275 prevented bone erosion and markedly delayed the onset of arthritis; at low doses, MS-275 strongly attenuated paw swelling, bone erosion, and bone resorption associated with RA. Furthermore, the therapeutic efficacy of MS-275 was also documented. After the onset of arthritis, it could stop the disease progression and joint destruction.. An anti inflammatory effect of MS-275 was also confirmed through its capacity to decrease serum IL-6 and IL-1β levels in the CIA induced mouse model. The anti-rheumatic activity of MS-275 was also confirmed through histological observation. No synovial hyperplasia, pannus formation, cartilage or bone destruction were observed in the high dose prophylactic intervention in mice.
Conclusion and implication: This study strongly supported HDACi as an innovative therapeutic strategy for RA.
British Journal of Pharmacology (2007) **150,** 862–872. doi:10.1038/sj.bjp.0707165; published online 26 February 2007

Keywords: rheumatoid arthritis; histone deacetylase inhibitor; suberoylanilide hydroxamic acid; MS-275

Abbreviations: BV/TV, bone volume/tibiae volume; CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; IFA, incomplete Freund's adjuvant; IL-6, interleukin-6; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MTX, methotrexate; RA, rheumatoid arthritis; SAHA, suberoylanilide hydroxamic acid; TNF-α, tumor necrosis factor-α

Introduction

Rheumatoid arthritis (RA) is a chronic symmetric polyarticular arthritis that mainly affects the small diarthrodial joints of the hands and feet (Lee and Weinblatt, 2001; Firestein, 2003). It is a fairly common disorder and occurs in 0.5–1% of the adult population, in a female/male ratio of 2.5:1 worldwide (Lee and Weinblatt, 2001; Firestein, 2003). Although the precise etiology remains unknown, RA is thought of as an autoimmune disease (ACR, 2002; Haringman *et al*., 2004). The inflamed synovium is central to the pathogenesis of RA (Lee and Weinblatt, 2001; Firestein, 2003) and formation of tumor-like synovial tissue, the so-called 'pannus' is a characteristic feature of RA (Lee and Weinblatt, 2001). Apart from destroying the affected joints, RA also has extra-articular effects in the body and osteoporosis is commonly associated with RA (Lee and Weinblatt, 2001; ACR, 2002). As a relapsing systemic disease, RA affects the physical functioning of patients, their psychological and social health eventually progresses to substantial disability (ACR, 2002; Bansback *et al*., 2005). Furthermore, RA patients

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Received 24 April 2006; revised 28 September 2006; accepted 21 November 2006; published online 26 February 2007

have a significantly higher incidence of fatality from cardiovascular diseases, infections and cancers than the general population (Bansback *et al*., 2005).

RA is an incurable disease and life-long therapy is required (Lee and Weinblatt, 2001; Bansback *et al*., 2005). Currently, four different classes of drugs, namely nonsteroid anti-inflammatory drugs (NSAIDs), analgesics, glucocorticoids and disease-modifying antirheumatic drugs (DMARDs) are used clinically (ACR, 2002). NSAIDs and analgesics do not alter the course of RA or prevent joint destruction; hence, they should not be used as the only treatment (ACR, 2002; Bansback *et al*., 2005). Serious adverse effects of long-term oral glucocorticoids are well known and these drugs have to be used carefully (ACR, 2002). The DMARDs are a large class of heterogeneous drugs, including sulfasalazine, antimalarials, penicillamine, gold, methotrexate (MTX), azathioprine, leflunomide and cyclophosphamide (ACR, 2002; Bansback *et al*., 2005). On the basis of its favorable efficacy/toxicity, low cost and established track record in RA treatment, MTX has become the standard of care for patients with moderate-to-severe RA (ACR, 2002; Bansback *et al*., 2005; Cronstein, 2005). However, issues pertaining to toxicity and adverse effects, together with the failure of achieving disease remission, prompt patients to discontinue or switch between DMARDs (ACR, 2002; Klinkhoff, 2004; Bansback *et al*., 2005). New classes of DMARDs, such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) inhibitors/modulators have recently become more popular. These drugs have a faster onset of action than the conventional DMARDs and have the prospect of significant inhibition of joint destruction and reduced toxicity (Klinkhoff, 2004; Bansback *et al*., 2005). However, high cost, continuous parenteral administration and an increased risk of infection are associated with TNF-α inhibitors (Bansback *et al*., 2005). Furthermore, nearly 25% of RA patients do not respond to anti-TNF-α therapy (Clair, 2002). Therefore, the RA therapies that are established so far are not ideal. Indeed, with roughly 165 million sufferers worldwide and significantly unmet clinical need (Green, 2000; Mount and Featherstone, 2005), there is a demand to develop cheaper therapies with enhanced disease-modifying effects and reduced toxicity or adverse effects.

The acetylation and deacetylation of histones play an important role in the control of gene expression (Johnstone, 2002; Piekarz and Bates, 2004). Acetylation, catalyzed by histone acetyl transferases, neutralizes the positive charge of the histones, decreases their binding affinity between DNA, and thereby facilitates the activation of gene transcription (Johnstone, 2002; Piekarz and Bates, 2004). In opposition to histone acetyl transferases, the deacetylation is mediated by histone deacetylases (HDACs), leading to transcription repression through chromatin condensation (Johnstone, 2002; Piekarz and Bates, 2004). HDAC inhibitors (HDACi) can modulate the activities of HDAC, and hence activate and/or repress subsets of genes (Johnstone, 2002; Piekarz and Bates, 2004). The antineoplastic activities of HDACi have been well studied. HDACi can induce cell-cycle arrest, cell differentiation and/or apoptotic cell death in various transformed cells (Johnstone, 2002; Chung *et al*., 2003; Piekarz and Bates, 2004). Furthermore, several HDACi are currently undergoing clinical investigation for oncological indications. Besides anticancer effects, HDACi also recently emerged as potent anti-inflammatory drugs (Blanchard and Chipoy, 2005). It was reported that HDACi can reduce the proinflammatory cytokines (e.g. TNF-α, IL-1β, IL-8, transforming growth factor-β), downregulate immune stimulators (e.g. IL-6, IL-10, CD154) and inhibit the production of nitric oxide, which also contributes to various inflammatory diseases (Huang *et al*., 1997; Mishra *et al*., 2001; Leoni *et al*., 2002, 2005; Chung *et al*., 2003; Blanchard and Chipoy, 2005; Leoni *et al*., 2005). Also, HDACi induced the expression of cancer cell-cycle inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, $p16^{INK4}$) and the tumor suppressor gelsolin, which suppresses the tumor-like transformed synovial cells (Chung *et al*., 2003; Blanchard and Chipoy, 2005; Hirokawa *et al*., 2005; Mukhopadhyay *et al*., 2006). Furthermore, in a pilot study, Chung *et al*. (2003) reported that topical phenylbutyrate or trichostatin therapy can reduce joint swelling, decrease subintimal mononuclear cell infiltration, inhibit synovial hyperplasia, suppress pannus formation and prevent cartilage or bone destruction in a rat adjuvant-induced arthritis model; similarly, beneficial results were obtained with FK-228 in a mouse autoantibody-mediated arthritis model (Nishida *et al*., 2004).

Suberoylanilide hydroxamic acid (SAHA) and MS-275 are two HDACi currently undergoing clinical investigations for various solid and hematological malignancies (Kelly *et al*., 2003, 2005; Johnstone, 2004; Ryan *et al*., 2005). Promising results including good oral bioavailability, linear pharmacokinetics, good tolerance and a broad range of antitumor activity were obtained from SAHA and MS-275 (Kelly *et al*., 2003, 2005; Johnstone, 2004; Ryan *et al*., 2005). In this study, we hypothesized that these two promising HDACi may also be effective in the management of RA. Antirheumatic activities of SAHA and MS-275 were assessed in rodent collagen-induced arthritis (CIA) models, and our results strongly support further investigation of HDACi as an innovative therapeutic strategy for RA.

Methods

Animals

DBA/1J mice (male, 8-weeks old) and Dark Agouti rats (female, 7–8-weeks old) were obtained from Harlan Laboratories (Gannat, France). Animal care was in accord with the European Guidelines for the humane use of animals in scientific research. The animals were kept on a 12 h light/dark cycle (0700–1900); food and water were provided *ad libitum*. All *in vivo* experiments were carried out in a specific pathogen-free facility (22°C).

Collagen-induced arthritis

One day before the experiment, bovine collagen type II solution (2 mg ml^{-1}) was prepared with 0.05 M acetic acid and stored at 4°C. Before the immunization, equal volumes of adjuvant (mouse: complete Freund's adjuvant (CFA), rat: incomplete Freund's adjuvant (IFA)) and bovine collagen type II were mixed by a homogenizer in a precooled glass bottle under ice-water bath. Extra adjuvant with prolonged

homogenization might be required if a stable emulsion was not formed. For mice, 0.1 ml of the emulsion was injected intradermally at the base of the tail of each mouse on day 1; lipopolysaccharide (LPS) booster injection (40 μg in 0.1 ml saline) was performed intraperitoneally on day 29. For rats, 0.2 ml of the emulsion was injected intradermally at the base of the tail of each rat on day 1, no booster injection was required. This immunization method was modified from published methods (Sims *et al*., 2004; Jou *et al*., 2005).

Study design

Mice prophylactic intervention with SAHA and MS-275. The prophylactic efficacy of SAHA and MS-275 was tested in the mouse CIA model. Seventy mice were randomly divided into seven groups of 10 mice. Mice in Group 1–6 were immunized on day 1, whereas Group 7 was used as the untreated control. Group 1 was treated with vehicle (0.1 ml 5% dimethyl sulfoxide (DMSO), once daily, s.c.); Group 2 was treated with the high dose of SAHA (50 mg kg^{-1}, once daily, s.c.); Group 3 was treated with the low dose of SAHA (5 mg kg^{-1}, once daily, s.c.); Group 4 was treated with the high dose of MS-275 (10 mg kg^{-1}, once daily, s.c.); Group 5 was treated with the low dose of MS-275 (3 mg kg^{-1}, once daily, s.c.); and Group 6 was treated with MTX (100 μg kg^{-1}, once daily, s.c.). Treatment was given daily on monday to friday, each week. The body weight was recorded weekly and the arthritic score was assessed three times weekly after the onset of arthritis. On day 40, 200 μl of blood was collected by retro-orbital puncture. Serum was prepared as described previously by Sims *et al*. (2004). On day 43, all mice were killed; hind paws were removed for the X-way analysis and the histological examination; the right tibiae were also removed for the measurement of bone volume/tibia volume (BV/TV).

Rats prophylactic intervention with SAHA. The prophylactic efficacy of SAHA was tested in the rat CIA model. Rats were randomly divided into six groups, Group 1 with 10 rats and Groups 2–6 with five rats each. All rats were immunized on day 1. Group 1 was treated with vehicle (0.5 ml 5% DMSO, once daily, s.c.); Group 2 was treated with the high dose of SAHA (50 mg kg^{-1}, once daily, p.o.); Group 3 was treated with the low dose of SAHA (5 mg kg^{-1}, once daily, p.o.); and Group 4 was treated with MTX (70 μg kg^{-1}, once daily, s.c.). The study was performed in the same way as the study in mice, except that the rats were killed on day 29.

Rats prophylactic and therapeutic interventions with MS-275. Both prophylactic and therapeutic efficacy of MS-275 were tested in the rat CIA model. One hundred rats were randomly divided into 10 equal groups. All rats were immunized on day 1 except for Group 10, which was used as the untreated control. Group 1 and 10 were treated with vehicle; Groups 2–4 were treated with preventive MS-275 dosing at 0.3, 1 or 3 mg kg^{-1} (once daily, s.c.); similarly, Group 5 was treated with preventive MTX dosing (70 μg kg^{-1}, once daily, s.c.). The MS-275 prophylactic study was carried out in the same way as the SAHA study in rats, except arthritic score was assessed daily after the onset of arthritis.

Groups 6–9 were treated with vehicle daily from day 1 to day 15 except saturdays and sundays. After day 15, most of the rats showed the onset of arthritis and Groups 6–8 were switched on day 16, to therapeutic MS-275 dosing at 1, 3 or 5 mg kg^{-1} (once daily, s.c.), respectively; Group 9 was treated with therapeutic MTX dosing (70 μg kg^{-1}, once daily, s.c.). Therapeutic dosing lasted from day 17 to day 28. Other treatments were exactly the same as Groups 1–5.

Clinical assessment of arthritis

Arthritis was scored as 5 grades according to a well-established method (Nishida *et al*., 2004). The inflammation of each of the four paws was ranked as follows: 0, no symptoms; 1, mild, but definite redness and swelling of one type of joint such as the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of two or more types of joints; 3, severe redness and swelling of the entire paw including digits; 4, maximally inflamed limb with involvement of multiple joints. Each limb was graded individually on a scale of 0–4 (maximum cumulative clinical arthritis score 16 per animal).

Change in body weight (%) after onset of arthritis

Clinically, loss of body mass is associated with RA (Rall and Roubenoff, 2004; Sims *et al*., 2004; Jou *et al*., 2005; Shelton *et al*., 2005). Thus, the changes in body weight after the onset of arthritis could be used as a nonspecific end point to evaluate the prophylactic efficacy of HDACi. The change in body weight (%) of each individual animal after the onset of arthritis was calculated as follows:

$$\text{Mice}: \frac{\text{BodyWeight(week6)} - \text{BodyWeight(week5)}}{\text{BodyWeight(week5)}} \times 100\%$$

$$\text{Rats}: \frac{\text{BodyWeight(week4)} - \text{BodyWeight(week3)}}{\text{BodyWeight(week3)}} \times 100\%$$

Bone erosion

X-ray photos were taken of the hind paws of each individual animal. A random identity number was assigned to each of the photo, and the severity of bone erosion was ranked by two independent scorers (without knowledge of the treatments) with the radiological score system as follows: 0, normal with intact bony outlines and normal joint space; 1, slight abnormality with any one or two of the exterior metatarsal bones showing slight bone erosion; 2, definite early abnormality with any 3–5 of the exterior metatarsal bones showing bone erosion; 3, medium destructive abnormality with all the exterior metatarsal bones as well as any one or two of the interior metatarsal bones showing definite bone erosions; 4, severe destructive abnormality with all the metatarsal bones showing definite bone erosion and at least one of inner metatarsal joints has been completely eroded leaving some bony joint outlines partly preserved; and 5, mutilating abnormality with no bony outlines could be deciphered. This scoring system was a

slight modification from previous publications (Salvemini *et al*., 2001; Bush *et al*., 2002; Sims *et al*., 2004; Jou *et al*., 2005).

X-ray microcomputed tomography analysis of proximal tibiae. Tibiae (left leg) were collected for histomorphometric analysis at the end of the treatment. Microcomputerized tomography (CT) scans of the metaphyseal tibia were performed at an isotropic resolution of 9 μm, to obtain trabecular bone structural parameters. Using a two-dimensional (2D) and three-dimensional model and a semiautomatic contouring algorithm, we determined three-dimensional BV, bone surface and the trabecular thickness. Three-dimensional images were obtained on a micro-CT scanner (μCT 20; Scanco Medical AG, Bassersdorf, Switzerland). The samples were stored in 70% ethanol until micro-CT scanning. In trabecular bone, basic structural metrics were measured using direct 2D morphometry (Ruegsegger *et al*., 1996; Kapadia *et al*., 1998).

Histology
After taking X-rays, the hind paws of mice were fixed in 10% phosphate-buffered formalin (pH 7.4), decalcified with rapid bone decalcifiant for fine histology (Laboratories Eurobio, Courtaboeuf Cedex B, France) and embedded in paraffin. To ensure extensive evaluation of the arthritic joints, at least four serial sections (5 μm thick) were cut and each series of sections are in between 100 μm. The sections were stained with hematoxylin and eosin (H&E). Histological examinations were performed under blindness. For each paw, three parameters, namely, synovial hypertrophy, cartilage damage and bone destruction were each assessed using a 4-point scale (0–3, where 0, normal; 1, mild; 2, moderate; and 3, maximal). These three scores were summed together as the histological score. The maximal histological score in the four series was used to evaluate the disease severity for that paw. This scoring system was modified from previously published methods (Salvemini *et al*., 2001; Bush *et al*., 2002; Sims *et al*., 2004; Jou *et al*., 2005).

IL-1β and IL-6 assays
Blood was drawn by retro-orbital puncture under general anesthesia of the mice at day 40. Serum IL-1β and IL-6 levels were measured by enzyme-linked immunosorbent assay using protocols supplied by the manufacturer (Biosource Europe, Nivelles, Belgium).

MS-275 and SAHA IC_{50} determination and HDAC assay on spleen samples
Human HDAC1, 2, 3 and 6 were isolated by reverse transcriptase-polymerase chain reaction and the nucleotide sequence was confirmed by DNA sequence analysis. Isolated sequences were flag-tagged subcloned into pcDNA3.1 vector (Invitrogen, Cergy-Pontoise, France) and expression was verified by Western blot using anti-flag antibodies.

COS-7 monkey kidney cells (ATCC) were plated in six-well plates and then transiently transfected with each of the HDAC expression constructs (1 μg total DNA) using DNA–lipid complex Fugene 6 (Roche Diagnostic, Meylan, France) according to the manufacturer's protocol. Control was carried out with empty vector. Forty-eight hours after transfection, cells were harvested and lysed with Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Five hundred micrograms of total protein was used to immunoprecipitate individual overexpressed HDAC proteins by mean of antiflag antibody coupled to Sepharose bead (Sigma, St Louis, MO, USA). HDAC activity was then measured in immunoprecipitates using Fluor de Lys kit (Tebu SA, Le Payrret-en-Yvelines, France). The IC_{50} values of MS-275 and SAHA were then determined on each of these preparations of HDAC.

In animals, HDAC activity was assessed in isolated cells from spleen. Just after killing the animals, spleens were removed, cut into small pieces and homogenized in a tissue homogenizer in phosphate-buffered saline buffer. Nuclear proteins were prepared using NE-PER nuclear extraction kits (Pierce, Rockford, IL, USA). HDAC activity was then measured in nuclear extract using Fluor de Lys kit (Biomol).

Statistics
Data are expressed as mean $\pm$ s.e.m. The two-tailed, independent sample *t*-test was used to analyze continuous data including BV/TV (unpaired) and change in body weight (paired). A nonparametric test (Wilcoxon two sample test) was used to analyze score data including arthritic severity score, radiological score, histological score. A *P*-value less than 0.05 was adopted to indicate statistical significant difference.

Materials
SAHA and MS-275 were synthesized by ProStrakan (Romainville, France). The purity of these compounds was greater than 95%. CFA and IFA were purchased from Difco (Detroit, MI, USA). Bovine collagen type II, LPS and MTX was obtained from Chondrex (Redmond, Washington DC, USA); Sigma (St Louis, MO, USA) and Acros Organics (Morris Plains, NJ, USA), respectively. All other reagents used were of reagent grade and all solvents were of analytical grade.

Results

Prophylactic efficacy of HDACi in mouse CIA model
HDACi was first tested in the mouse CIA model. The systemic arthritis was induced with bovine collagen type II and CFA, plus a LPS booster injection on day 29 in DBA/1J mice. We assessed the severity of the arthritis with a well-established arthritic score system (Nishida *et al*., 2004). In this study, the incidence of arthritis was 100% (all the mice responded with an arthritic score >1) if the mice were not treated with antirheumatic agents. Thus, the difference in the severity of arthritis could be attributed to the pharmacological actions of HDACi or MTX.

In the mouse model, arthritic scores are shown in Figure 1a. The arthritis progressed rapidly in vehicle-treated

mice and the maximal clinical symptoms were achieved in about 1 week after the onset of arthritis. MTX (100 $\mu g\,kg^{-1}$) only exhibited very weak inhibitory effects on the onset and development of arthritis. SAHA exhibited moderate anti-rheumatic activities and its pharmacological actions appeared to be dose dependent. Although SAHA did not delay the onset of arthritis, it decreased the severity of paw swelling in comparison with the vehicle treatment. The arthritic scores of the mice treated with high-dose SAHA (50 $mg\,kg^{-1}$) were lower than those of the vehicle group and resulted in a 40–50% decrease in the maximal arthritic scores. At the low dose (5 $mg\,kg^{-1}$), SAHA attenuated slightly the severity of paw swelling. In contrast to SAHA, MS-275 showed dramatic antirheumatic activities. High prophylactic dosing of MS-275 (10 $mg\,kg^{-1}$) markedly delayed the onset of CIA. Only two of 10 mice developed arthritis immediately after the LPS booster injection; moreover, the swelling diminished rapidly and disappeared within 1 week. Although the low prophylactic dose (3 $mg\,kg^{-1}$) of MS-275 could not block the onset of CIA, strong protective effects were observed. The clinical scores of the mice treated with low doses of MS-275 (3 $mg\,kg^{-1}$) were much lower than those of the vehicle group, resulting in a decrease of more than 50% in the maximal arthritic scores.

The body weight of the mice was recorded weekly. All the mice gained body growth with similar rate before the onset of arthritis (weeks 1–5), indicating both SAHA and MS-275 were well tolerated at our tested doses. The changes in body weight after the onset of arthritis were used as a nonspecific end point to evaluate the prophylactic efficacy of HDACi and our results are shown in Figure 1b. Significant weight loss occurred in all arthritis-bearing mice in our study. The vehicle group suffered from serious weight loss (about 15% in 1 week) and treatment with SAHA (5 $mg\,kg^{-1}$) or MS-275 (10 $mg\,kg^{-1}$) had some beneficial effects in the relief of weight loss.

As shown in Figure 1c, serum IL-6 in intact mice was undetectable ($<0.05\,pg\,ml^{-1}$), but after the induction of CIA, IL-6 increased to about 35 $pg\,ml^{-1}$. In mice treated with 5 or 50 $mg\,kg^{-1}$ of SAHA, serum IL-6 was not significantly decreased. In contrast, animals treated with 3 or 10 $mg\,kg^{-1}$ of MS-275, exhibited a marked decrease of serum IL-6 level by 59 and 93%, respectively. In the group treated with MTX, serum IL-6 level was also significantly decreased by about 90%.

Similar results were observed with the IL-1β assays. In our mouse CIA model, the raised serum IL-1β levels in CIA mice were markedly decreased (by about 89%) after MTX treatment (Figure 1d). In mice treated with 50 $mg\,kg^{-1}$ SAHA, serum IL-1β levels were not significantly decreased, compared with the untreated mouse CIA group. However, in mice treated with 3 or 10 $mg\,kg^{-1}$ MS-275, serum IL-1β

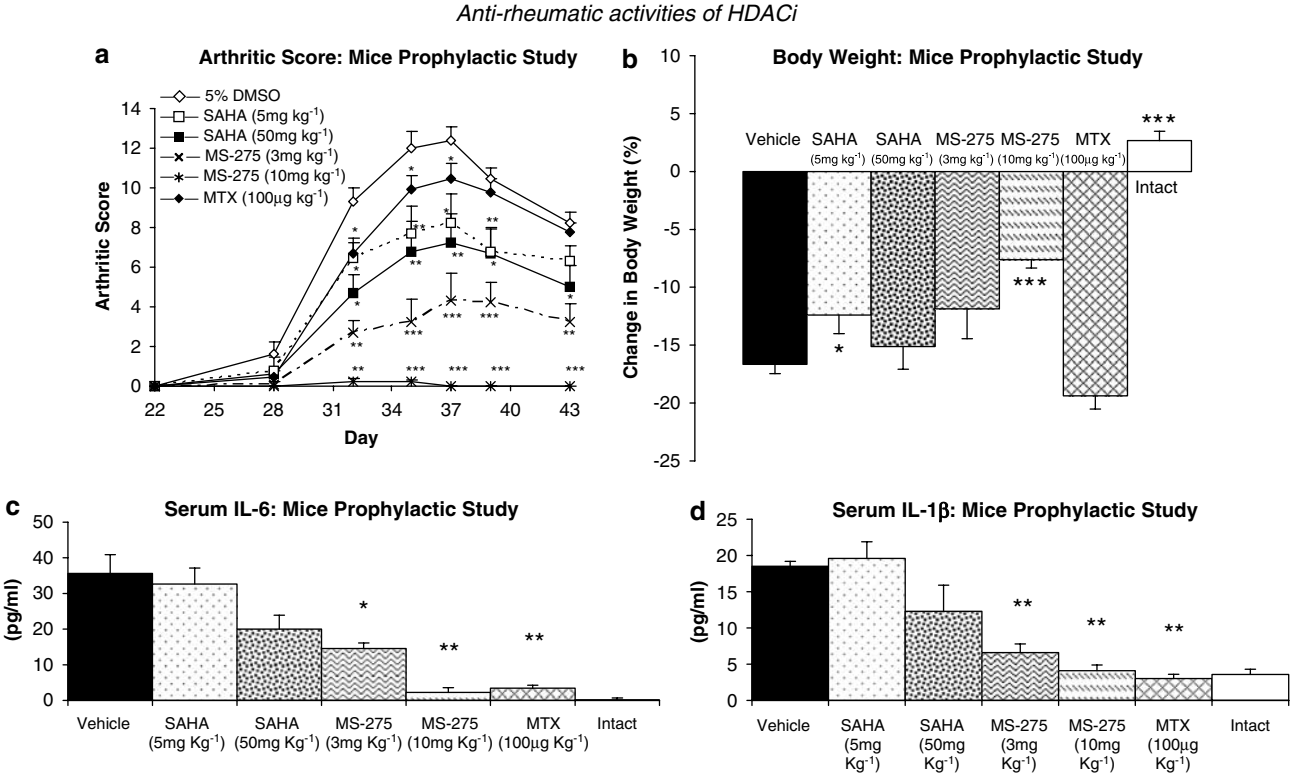

Figure 1 Prophylactic efficacy of HDACi in mice. CIA was induced as described in the Methods section. Mice were treated with vehicle, prophylactic doses of SAHA, MS-275 or MTX (5 days per week). (**a**) Arthritic score. Arthritic score was assessed three times weekly after the onset of arthritis. (**b**) Body weight. The change in body weight was calculated from the body weight of each individual mouse at weeks 5 and 6. (**c**) Serum IL-6 levels were assessed 3 days before termination of the experiment at day 43. (**d**) Serum IL-1β levels were assessed 3 days before day 40. Symbols represent mean value with s.e.m. shown. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as compared with vehicle treatment; $n=10$ for each group. (Clinical score: Wilcoxon two sample test; change in body weight: two-tailed independent sample *t*-test.)

decreased by 65–70%, compared with that in the untreated CIA group.

Marginal bone erosion is one of the major features of joint damage caused by RA (Gravallese and Goldring, 2000; Lee and Weinblatt, 2001). Joint narrowing was observed in most of the arthritis-bearing mice (photo not shown). The radiological score data are listed in Table 1. All mice treated with vehicle suffered from very serious erosion with a radiological score of about 4 (the maximal score is 5). Even at a low dose (3 mg kg^{-1}), MS-275 exhibited strong antierosion effects and only slight abnormalities were observed; furthermore, no bone erosion was observed in mice treated with the higher dose of MS-275 (10 mg kg^{-1}). SAHA also exhibited some bone protective effects at both doses. Again, MTX only exhibited very weak protective activity against bone erosion. The radiological scores also correlated well with the arthritic scores, as shown in Figure 1a.

Table 1 Effects of HDACi on bone destruction in prophylactic study in mice[a]

	Radiological score	*BV/TV (%)*
Vehicle	3.9±0.4	1.998±0.228
SAHA (5λmg kg^{-1})	2.4±0.4*	2.609±0.421
SAHA (50λmg kg^{-1})	2.4±0.5*	1.439±0.358
MS-275 (3λmg kg^{-1})	0.9±0.5**	4.889±0.823**
MS-275 (10λmg kg^{-1})	0.0±0.0***	6.193±0.448***
MTX (100 μg kg^{-1})	2.7±0.4*	2.951±0.306*
Intact	0.0±0.0***	11.386±0.382***

Abbreviations: BV/TV, bone volume/tibial volume; HDAC, histone deacetylase inhibitor; MTX, methotrexate.
[a]Data are expressed as mean±s.e.m.; *$P<0.05$, **$P<0.01$, ***$P<0.001$, as compared with vehicle treatment ($n=10$) (radiological score: Wilcoxon two-sample test; BV/TV: two-tailed independent sample *t*-test).

Systemic bone loss is also a feature of RA. In this study, we assessed bone loss by measuring the BV/TV ratio in the right tibiae with a micro-CT and the results are shown in Table 1. All animals in vehicle treatment suffered very serious bone loss, with the BV/TV ratio after vehicle treatment being only about 20% of the BV/TV in nonarthritic, intact animals. MS-275 had dose-dependent protection in bone loss; MTX had weak protective effects, whereas no significant protective effects of SAHA were observed. Again, although the higher dose of MS-275 almost prevented the onset of clinically apparent arthritis, significant bone loss was also observed in comparison with nonarthritic intact mice, indicating that these animals still responded to the immunization.

RA is well characterized with synovial hyperplasia, pannus formation, and cartilage and bone destruction in the joint. Such rheumatic features were very apparent in mice that received the vehicle treatment (Figure 2a). To confirm the protective effects of HDACi, histological analysis was also carried out on the mouse hind paws. Each specimen was ranked with a histological score according to the severity in synovial hyperplasia, cartilage damage and bone destruction. The results of such histological scoring are shown in

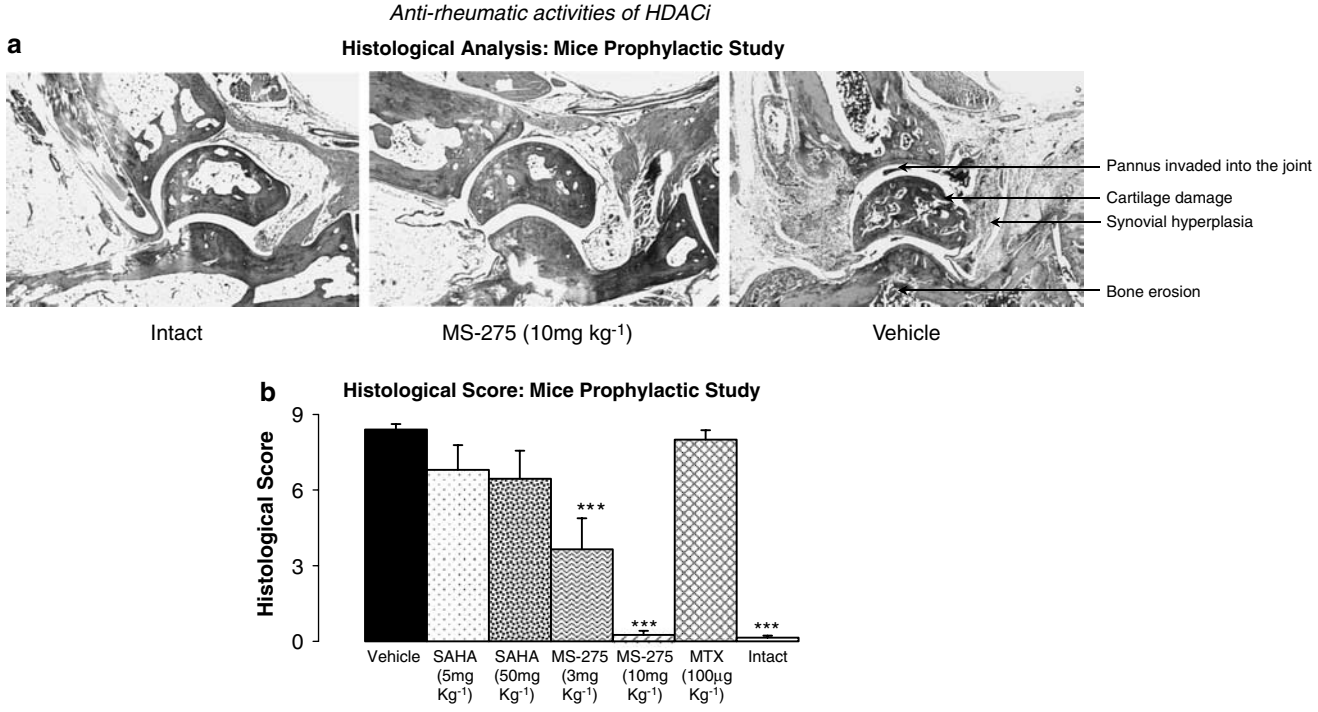

Figure 2 Histological analysis of CIA in mice. The histological analysis was performed with mice hind paw sections stained by H&E. (**a**) Metatarsal joints from a symptom-free mouse (intact), arthritis-bearing mouse (vehicle) and a mouse treated with MS-275 (10 mg kg^{-1}) (magnification ×125). Synovial hyperplasia, pannus formation and bone and joint destruction were observed in the metatarsal joint of the mouse receiving vehicle treatment. (**b**) Histological score. Mice were treated with vehicle or prophylactic doses of SAHA, MS-275 or MTX. The histological score was assessed according to the severity of synovial hyperplasia, bone damage and cartilage destruction. Symbols represent mean value with s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$; $n=10$ for each group (Wilcoxon two-sample test), as compared with vehicle treatment of each study.

Figure 2b. The vehicle treatment group had serious synovial hyperplasia, cartilage damage and bone destruction with a cumulative histological score close to 8 (the maximal score is 9), whereas no noticeable synovial hyperplasia, cartilage damage and bone destruction was observed in high-dose MS-275 treatment (Figure 2a and b). Furthermore, our histological analysis also found that about 50% of the hind paw specimens from the mice treated with low-dose MS-275 were symptom-free (data not shown). However, no significant protective effects of SAHA or MTX were observed.

To compare the potency of MS-275 and SAHA as HDACi, we determined the IC_{50} of these compounds *in vitro*, in a general HDAC activity assay as well as in assays with specific HDAC isoforms. The results shown in Table 2 demonstrate that, in the general HDAC assay, both MS-275 and SAHA had similar IC_{50} values. However in specific HDAC assays, MS-275 displayed a profile that was distinct from that of SAHA. MS-275 was more potent against HDAC1 and HDAC3, whereas SAHA was more potent against HDAC2. As expected, both inhibitors were inactive in the HDAC6 assay.

Finally, to make sure that MS-275 and SAHA did inhibit HDAC activity when given *in vivo*, we measured HDAC activity in nuclear extracts from spleen cells harvested from either vehicle, MS-275 ($10\,mg\,kg^{-1}$) or SAHA ($50\,mg\,kg^{-1}$)-treated animals. The results (Table 3) clearly show that both MS-275 and SAHA significantly reduced HDAC activity. Under these conditions, MS-275 was more potent than SAHA.

Prophylactic efficacy of HDACi in rat CIA model

Clinically evident arthritis was observed in Dark Agouti rats on about day 14. Again, the induction of CIA was very successful with 100% incidence in the rats not treated with antirheumatic agents. The arthritic scores and changes in body weight data are shown in Figure 3. As in the mouse prophylactic study, both MS-275 and SAHA exhibited dose-dependent antirheumatic activities. At the high dose ($3\,mg\,kg^{-1}$), MS-275 markedly reduced the onset of arthritis (Figure 3a); at the low dose ($1\,mg\,kg^{-1}$), it dramatically decreased the severity of arthritis, whereas at $0.3\,mg\,kg^{-1}$, no significant antirheumatic effects were observed. SAHA also exhibited some antirheumatic activities, although it was not as potent as MS-275. At both doses (50 and $15\,mg\,kg^{-1}$), SAHA decreased the arthritic score. The protective effects of MS-275 and SAHA in bone erosion were also observed in rats. At $3\,mg\,kg^{-1}$, MS-275 prevented bone erosion; at $1\,mg\,kg^{-1}$, it strongly suppressed bone erosion (radiological score: MS-275 (1.5 ± 0.4) vs vehicle (4.5 ± 0.2), $P<0.001$). Similarly, high-dose SAHA ($50\,mg\,kg^{-1}$) attenuated bone erosion (radiological score: SAHA (1.9 ± 0.8) vs vehicle (4.3 ± 0.1), $P<0.05$). Furthermore, both MS-275 and SAHA were able to decrease the bone resorption enhanced by RA (data not shown). MTX ($70\,\mu g\,kg^{-1}$) had very strong prophylactic efficacy in rats. It markedly inhibited the onset of arthritis, prevented the bone erosion and significantly reversed the enhanced bone resorption associated with RA (data not shown). The prophylactic efficacy of MTX ($70\,\mu g\,kg^{-1}$) was comparable with the activities of MS-275 ($3\,mg\,kg^{-1}$).

Table 2 Profile of inhibition of specific HDACs by MS-275 or SAHA

	General HDAC	*HDAC1*	*HDAC2*	*HDAC3*	*HDAC6*
MS-275	0.095 ± 0.11	1.04 ± 0.13	0.68 ± 0.24	0.12 ± 0.09	>50
SAHA	0.088 ± 0.08	2.35 ± 0.21	0.33 ± 0.12	0.86 ± 0.21	>50

Abbreviations: HDAC, histone deacetylase inhibitor.
Data shown are IC_{50} values (μM; mean $\pm$ s.e.m.) obtained from four assays.

Table 3 MS-275 and SAHA inhibit HDAC activity in extracts of spleen cells from mice treated *in vivo*[a]

	General HDAC assay
Vehicle	2345 ± 63
MS-275 ($10\,mg\,kg^{-1}$)	1447 ± 183* (38%)
SAHA ($50\,mg\,kg^{-1}$)	1972 ± 30* (16%)

[a]Results shown are fluorescent units of activity per $10\,\mu g$ nuclear protein (means $\pm$ s.e.m., from five assays). In parentheses, results are shown as % inhibition relative to the value after vehicle treatment.
*$P=0.05$ vs vehicle; means $\pm$ s.e.m., from five mice.

Therapeutic efficacy of MS-275 in rat CIA model

As MS-275 exhibited strong prophylactic activities in both mice and rats, we decided to assess its therapeutic efficacy, which is more relevant to the clinical management of RA. On day 17, when 90% of the rats had onset of the arthritis, treatment with MS-275 was started with 1, 3 or $5\,mg\,kg^{-1}$. Treatment with MTX ($70\,\mu g\,kg^{-1}$) in a group of animals was also performed as a positive control (Figure 4a. The baseline levels of arthritic severity of the rats in the therapeutic study were similar with a mean arthritic score around 4.7 for all groups. MS-275 at $1\,mg\,kg^{-1}$ had no statistically significant therapeutic effects, although the arthritic score was slightly lower than in the control group. At a dose of 3 or $5\,mg\,kg^{-1}$, MS-275 blocked the further development of arthritis and the arthritic score diminished after 4–5 doses of MS-275. After 1 week of treatment, the arthritis tended to be stabilized at a level with an arthritic score of 4–5. The therapeutic efficacy of MS-275 at $5\,mg\,kg^{-1}$ was higher than at $3\,mg\,kg^{-1}$, showing dose-dependency. At $70\,\mu g\,kg^{-1}$, MTX had significant therapeutic effects and it attenuated the arthritic score in comparison with the vehicle.

Protective effects of MS-275 on bone erosion were also observed. Joint narrowing and serious bone erosion were observed in the rats, which received the vehicle treatment (Figure 4b). In contrast to the vehicle treatment, only slight abnormalities were observed in the rats treated with high therapeutic doses of MS-275 ($5\,mg\,kg^{-1}$) (Figure 4b). In contrast, MTX did not exhibit significant protective activities in bone erosion. The rats that received MTX therapeutically also developed bone erosion comparable with the vehicle group. To quantify the damage in marginal bone erosion owing to RA, we ranked the X-ray photos with the radiological score system and the results are shown in Figure 4c. Again, dose-dependent antierosive activities of MS-275 were observed. The radiological score data (Figure 4c) correlated well with the arthritic score data (Figure 4a). Similarly, MS-275 therapeutics ($5\,mg\,kg^{-1}$) but

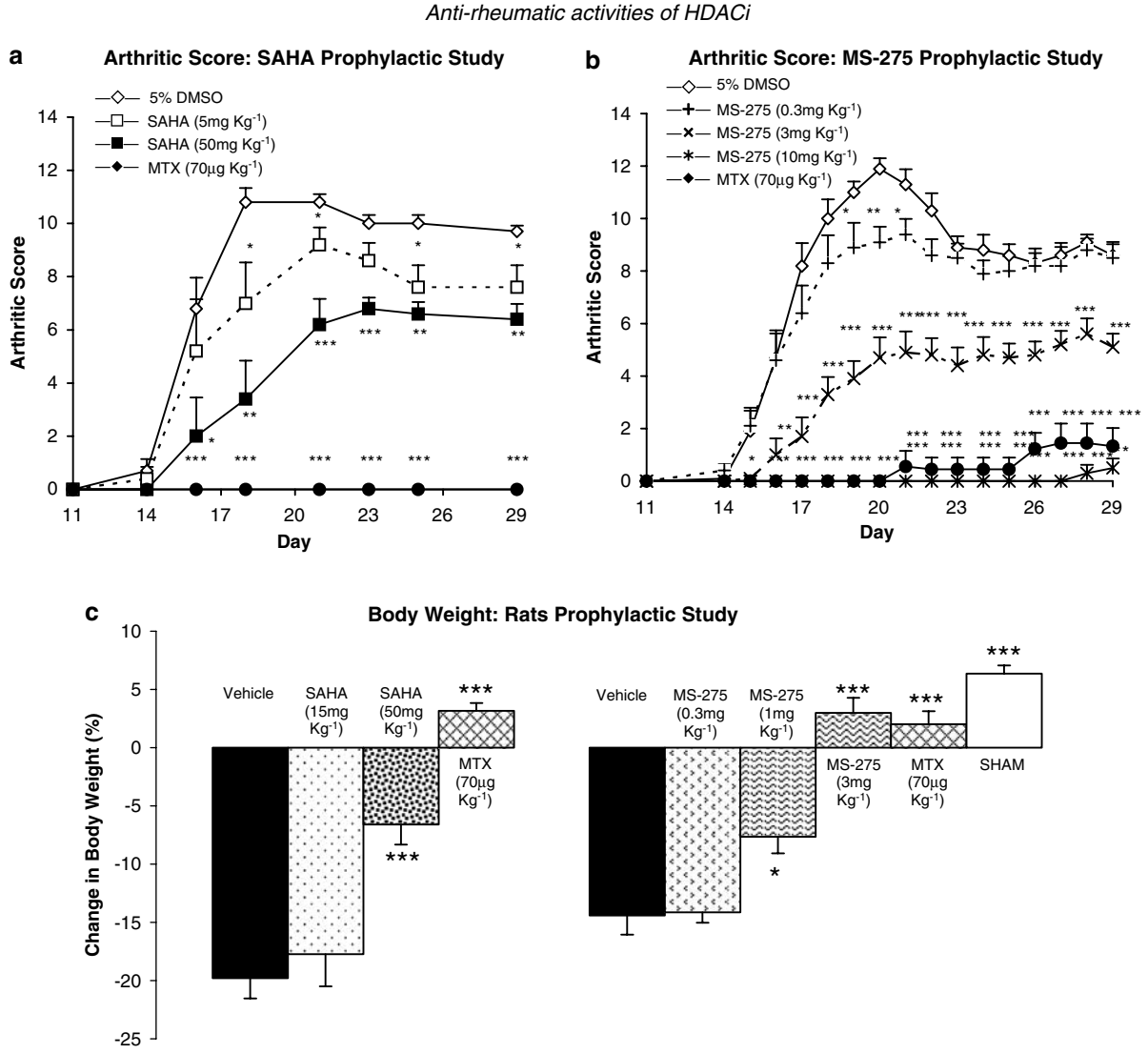

Figure 3 Prophylactic efficacy of HDACi in rats. Arthritis was induced as described in the Methods section. Rats were treated with vehicle, prophylactic doses of SAHA, MS-275 or MTX (5 day per week). (**a**) Arthritic score: SAHA prophylactic study. Arthritic score was assessed three times weekly after the onset of arthritis. (**b**) Arthritic score: MS-275 prophylactic study. Arthritic score was assessed daily after the onset of arthritis. (**c**) Body weight. The change in body weight was calculated from the body weight of each individual rat at weeks 3 and 4. Symbols represent mean value with s.e.m. shown. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as compared with vehicle treatment; see methods section for details of group sizes (5–10) (clinical score: Wilcoxon two sample test; change in body weight: two-tailed independent sample *t*-test).

not MTX therapeutics ($70\,\mu g\,kg^{-1}$) attenuated the bone resorption accelerated by RA (Figure 4d).

Discussion

In this study, antirheumatic activities of SAHA and MS-275 were documented. SAHA exhibited moderate antirheumatic activities. Although it could not prevent the development of arthritis, SAHA suppressed paw swelling, decreased bone erosion in both mice and rats and slightly reduced the RA-induced bone resorption in rats. In high-dose prophylactic studies, MS-275 significantly inhibited the onset of arthritis; and no characteristic signs of RA, including synovial hyperplasia, pannus formation, cartilage damage and bone destruction were observed in histological analysis. Systemic bone loss is also a feature of RA and it could lead to osteoporosis (Gravallese and Goldring, 2000; Lee and Weinblatt, 2001). Osteoclastogenesis secondary to the inflammatory process is evident in animal models of RA such as CIA (Gravallese and Goldring, 2000; Saidenberg-Kermanac'h *et al*., 2004).

At low dosages, MS-275 strongly attenuated paw swelling, bone erosion and bone resorption associated with RA. Prophylactic treatment with MS-275 resulted in reduced levels of circulating IL-1β and IL-6 in the mouse CIA model, but high doses of SAHA did not inhibit serum levels of these inflammatory cytokines. The present study appears to be one

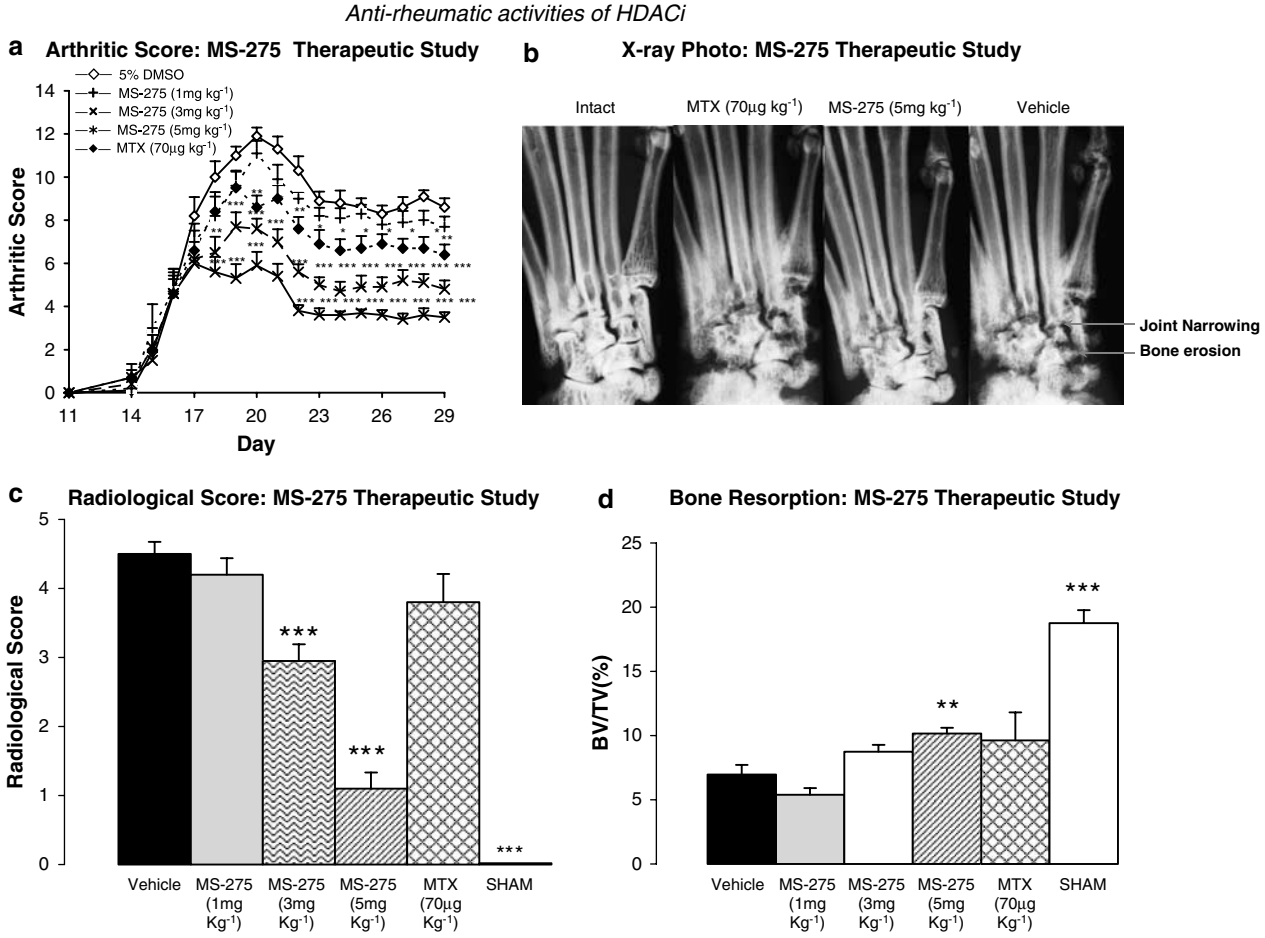

Figure 4 Therapeutic efficacy of HDACi in rats with CIA. Rats received daily treatment of vehicle from days 1 to 15 (5 days per week); daily therapeutic intervention of MS-275 or MTX lasted from day 17 to day 28. (**a**) Arthritic score. Arthritic score was assessed daily after the onset of arthritis. (**b**) X-ray photo. The X-ray photo was taken of the hind paws of the rats. Arthritis-bearing rats suffered serious bone erosion and joint narrowing. (**c**) Radiological score. The severity of bone erosion was assessed with the radiological score system. (**d**) Bone resorption. The bone resorption was measured with micro-CT scan and the BV/TV value was recorded. Symbols represent mean value with s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $n=10$ all groups (radiological score: Wilcoxon two-sample test; BV/TV: two-tailed independent sample *t*-test), as compared with vehicle treatment.

of the first demonstrating the possible anti-inflammatory effects of HDACi *in vivo*. Furthermore, after the onset of arthritis, MS-275 could halt the disease progression and joint destruction, and the therapeutic efficacy of MS-275 was even greater and faster in onset than that of MTX, the first-line drug used in the management of RA. As suggested by two previous studies (Chung *et al*., 2003; Nishida *et al*., 2004), our findings strongly support the concept of inhibition of HDAC as a therapeutic strategy for RA.

The anti-rheumatic mechanisms of action of HDACi remain largely unknown. Originally designed for oncological indications, HDACi are generally thought to exhibit their antineoplastic activities through alteration of gene expression (Johnstone, 2002, 2004; Piekarz and Bates, 2004). Previous analysis of gene expression profiles estimated that between 2 and 9% of the genome might be regulated by HDACi, with equal numbers of tested genes activated or suppressed (Johnstone, 2002; Blanchard and Chipoy, 2005). It was reported that HDACi have multiple targets involved in RA. HDACi reduce the expression of some proinflammatory cytokines, especially TNF-α and IL-1 (Leoni *et al*., 2002; Chung *et al*., 2003; Johnstone, 2004; Nishida *et al*., 2004; Blanchard and Chipoy, 2005; Leoni *et al*., 2005); induce growth arrest and/or apoptosis of the transformed RA synovial cells through the upregulation of the cell-cycle inhibitor p21 (Waf/Cip1) (Chung *et al*., 2003; Nishida *et al*., 2004; Jungel *et al*., 2006); inhibit angiogenesis (Deroanne *et al*., 2002; Mie Lee *et al*., 2003; Kim *et al*., 2004; Michaelis *et al*., 2004; Qian *et al*., 2004); and decrease the expression of metalloproteinases (Mort, 2005; Young *et al*., 2005), all of which play an important role in bone and cartilage destruction in RA. All these mechanisms could provide beneficial effects in RA management. However, the exact anti-inflammatory mechanisms of SAHA and MS-275 are still unknown and further mechanistic investigations are warranted.

In this study, the antirheumatic activities of MS-275 appeared to be more powerful than SAHA. This could be due to drug delivery or pharmacokinetic properties. However, it is also possible that the specificities of SAHA and

MS-275 for different families of HDACs underlie the difference in antirheumatic efficacy. Mammalian HDACs are classified into three classes: Class I HDACs (HDAC 1, 2, 3 and 8), homologues of yeast RPD3 are located exclusively in the cell nucleus; Class II HDACs (HDAC 4, 5, 6, 7, 9 and 10), homologues of yeast HDA1 are located in the nucleus and can migrate between cytoplasm and nucleus; Class III HDACs (Sirt 1–7), homologues of yeast silent information regulator 2 (Sir2), form a structurally distinct class of nicotinamide adenine dinucleotide (NAD)-dependent enzymes (Johnstone, 2002; Blanchard and Chipoy, 2005). Both Classes I and II HDACs have tissue-specific expression profiles and HDAC 1, 2 and 3 are ubiquitously expressed in various immune tissues (Blanchard and Chipoy, 2005). Furthermore, various previous studies suggested that Class I HDACs, most presumably HDAC 1, 2 and 3, could have inflammatory properties (Blanchard and Chipoy, 2005). SAHA inhibits Classes I and II HDACs with $IC_{50} = 10$–300 nM, whereas MS-275 preferentially inhibits HDAC 1 ($IC_{50} = 300$ nM) vs HDACs 3 ($IC_{50} = 8\,\mu$M), and has no inhibitory activity on HDAC 8 (Blanchard and Chipoy, 2005). The superior anti-inflammatory effects of MS-275 might be owing to its specificity on Class I HDACs, especially HDAC 1. As MS-275 has high specificity on HDAC 1 and exhibited good efficiency in RA, the genes controlled by Class I HDAC, presumably by HDAC 1 may play an important role in the pathogenesis of RA. Furthermore, enhanced antirheumatic efficacy of an HDACi with high specificity of Class I HDAC, particularly HDAC 1 is being postulated. The results from two previous studies support this hypothesis. A single intravenous dose of FK-228 (2.5 mg kg^{-1}), an inhibitor of Class I HDAC nearly eliminated the clinical symptoms even after the onset of arthritis in a mouse autoantibody-mediated arthritis model (Nishida *et al*., 2004), whereas daily topical treatment of phenylbutyrate (200 mg per paw b.i.d.) or trichostatin (100 mg per paw b.i.d.), inhibitors for Classes I and II HDAC could not prevent the development of arthritis and only diminished the local paw swelling in a rat adjuvant-induced arthritis model (Chung *et al*., 2003). However, further investigations with other HDACi are warranted to explore the relations between HDAC specificity and antirheumatic efficacy.

Though both SAHA and MS-275 were well tolerated in this study, their adverse effects in humans have been reported in several initial trials (Kelly *et al*., 2003, 2005; Ryan *et al*., 2005). Originally designed for oncological applications, such toxicities might not be crucial when taking into consideration their therapeutic effects and the high mortality rate of cancer. However, RA is a chronic disease and life-long therapy is required (Lee and Weinblatt, 2001; Bansback *et al*., 2005). It is well known that RA leads to body mass loss, probably due to inflammatory cytokines, pain, loss of appetite, increased energy expenditure and enhanced protein catabolism (Argiles and Lopez-Soriano, 2002; Rall and Roubenoff, 2004; Walsmith *et al*., 2004; Shelton *et al*., 2005). Such adverse effects of HDACi might be a more serious issue for application to RA. Clinical studies however indicate that the toxicity of antirheumatic drugs is one of the major reasons that prompt patients to switch between different therapies (ACR, 2002; Klinkhoff, 2004; Bansback *et al*., 2005). Thus, the development of new generations of HDACi with low toxicities is needed. Altogether, our data suggest that the differences between MS-275 and SAHA in terms of their inhibitory profiles *in vitro* and potencies *in vivo* could in part account for differences in efficacy of these two inhibitors in our RA model. The results of this study suggest that higher HDAC specificity might result in better antirheumatic efficiency (MS-275 vs SAHA). Similarly, improvement of the specificity for particular forms of HDACs may decrease interference with expression of other genes and hence reduce clinical toxicities or side effects.

In the current study, potent therapeutic efficacy of MS-275 was demonstrated in rodent CIA models. However, MS-275 could not reverse the damage due to arthritis; and the arthritic score, radiological score and BV/TV value did not return to symptom-free level. Such findings were in good agreement with the clinical situation. It is well known that the destruction caused by RA is irreversible and RA is an incurable disease (Quinn *et al*., 2001). All current clinical therapies are thus aimed at controlling the disease progression and the damage due to RA (ACR, 2002). Therefore, early diagnosis and immediate therapy with disease-modifying antirheumatic drugs are extremely important for the clinical management of RA (Quinn *et al*., 2001). We believe that immediate systemic treatment with HDACi, which demonstrate rapid and strong disease modifying effects, after early diagnosis of RA will provide a new strategy for the management of RA.

Acknowledgements

H-SL, C-YH, H-YC, Y-YL and H-PH were awardees of the Training and Attachment Program from the Economy Development Board, Republic of Singapore.

Conflict of interest

This authors state no conflict of interest.

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