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A New Class of Potent Matrix Metalloproteinase 13 Inhibitors for Potential Treatment of Osteoarthritis

Evidence of Histologic and Clinical Efficacy Without Musculoskeletal Toxicity in Rat Models

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Objective. **Matrix metalloproteinases (MMPs) have long been considered excellent targets for osteoarthritis (OA) treatment. However, clinical utility of broad-spectrum MMP inhibitors developed for this purpose has been restricted by dose-limiting musculoskeletal side effects observed in humans. This study was undertaken to identify a new class of potent and selective MMP-13 inhibitors that would provide histologic and clinical efficacy without musculoskeletal toxicity.**

Methods. **Selectivity assays were developed using catalytic domains of human MMPs. Freshly isolated bovine articular cartilage or human OA cartilage was used in in vitro cartilage degradation assays. The rat model of monoiodoacetate (MIA)–induced OA was implemented for assessing the effects of MMP-13 inhibitors on cartilage degradation and joint pain. The surgical medial meniscus tear model in rats was used to evaluate the chondroprotective ability of MMP-13 inhibitors in a chronic disease model of OA. The rat model of musculoskeletal side effects (MSS) was used to assess whether selective MMP-13 inhibitors have the joint toxicity associated with broad-spectrum MMP inhibitors.**

Results. **A number of non–hydroxamic acid–containing compounds that showed a high degree of potency for MMP-13 and selectivity against other MMPs were designed and synthesized. Steady-state kinetics experiments and Lineweaver-Burk plot analysis of rate versus substrate concentration with one such compound, ALS 1-0635, indicated linear, noncompeti-**

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tive inhibition, and Dixon plot analysis from competition studies with a zinc chelator (acetoxyhydroxamic acid) and ALS 1-0635 demonstrated nonexclusive binding. ALS 1-0635 inhibited bovine articular cartilage degradation in a dose-dependent manner (48.7% and 87.1% at 500 n*M* and 5,000 n*M*, respectively) and was effective in inhibiting interleukin-1α– and oncostatin M–induced C1,C2 release in human OA cartilage cultures. ALS 1-0635 modulated cartilage damage in the rat MIA model (mean ± SEM damage score 1.3 ± 0.3, versus 2.2 ± 0.4 in vehicle-treated animals). Most significantly, when treated twice daily with oral ALS 1-0635, rats with surgically induced medial meniscus tear exhibited histologic evidence of chondroprotection and reduced cartilage degeneration, without observable musculoskeletal toxicity.

Conclusion. **The compounds investigated in this study represent a novel class of MMP-13 inhibitors. They are mechanistically distinct from previously reported broad-spectrum MMP inhibitors and do not exhibit the problems previously associated with these inhibitors, including selectivity, poor pharmacokinetics, and MSS liability. MMP-13 inhibitors exert chondroprotective effects and can potentially modulate joint pain, and are, therefore, uniquely suited as potential disease-modifying osteoarthritis drugs.**

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases. They are synthesized as inactive zymogens and activated primarily extracellularly through cleavage of their pro domains by other enzymes, including activated MMPs (1). As a family, MMPs can cleave virtually any component of the extracellular matrix (1), and thereby facilitate cell migration and affect cellular signaling and functions. In addition to cleaving the structural components of the extracellular matrix, MMPs are involved in processing of growth factors and their receptors, cytokines, chemokines, adhesion molecules, and a variety of other enzymes (1). Thus, by altering the structure and function of a diverse group of substrates, MMPs can potentially mediate numerous physiologic and pathologic processes.

MMPs have long been considered excellent targets for treatment of osteoarthritis (OA), and inhibition of their activity has proven to be efficacious in a variety of models of experimentally induced as well as spontaneously occurring disease (2–5). However, the clinical utility of broad-spectrum MMP inhibitors developed for treatment of OA has been restricted by dose- and duration-dependent musculoskeletal side effects in humans (joint stiffness, inflammation, pain in the hands, arms, and shoulders termed "musculoskeletal syndrome" [MSS]). MSS is generally reversible upon cessation of drug treatment (6,7). No specific MMP has been implicated, and it is believed that nonselective inhibition of multiple MMPs is the primary cause of this toxicity (8). Therefore, current drug development strategies for treatment of OA are focused on inhibition of specific MMPs implicated in the disease process.

Approximately 20 million Americans 25–74 years of age (12% of the US population) are estimated to have OA (9). OA is the leading cause of joint pain and disability in middle-aged and elderly patients. It is characterized by progressive loss of articular cartilage that eventually leads to denudation of the joint surface. The cartilage loss observed in OA is the result of a complex process involving degradation of various components of the cartilage matrix; chief among them are the cartilage-specific type II collagen (CII) and aggrecan. While the loss of aggrecan is thought to be an early and reversible process, degradation of CII is considered irreversible and a key step in the loss of structural and functional integrity of cartilage (10). CII is a triple-helical protein that is resistant to degradation by most proteases but is readily cleaved by mammalian collagenases (MMPs 1, 8, and 13).

Of the various MMPs, MMP-13 has attracted the most attention as having a major role in OA. MMP-13 is specifically expressed in the cartilage of human OA patients, and is not present in normal adult cartilage (11–15). It is the major collagenase in OA cartilage and has the highest activity against CII (16,17). It cleaves the triple-helical collagen at a unique site, resulting in a three-quarter–length and a one-quarter–length product (11,12,18). Data on MMP-13–transgenic animals indicate that MMP-13 induces joint abnormalities characteristic of human OA (19). Moreover, semiselective MMP-13 inhibitors block degradation of human OA cartilage cultures (20). Recently, the chondroprotective effect of MMP-13–selective inhibitors was demonstrated in the rabbit surgical model of OA (21). Taken together, these data indicate that MMP-13 plays a pivotal role in the cartilage degradation observed in OA.

We have identified a new class of potent inhibitors that block cartilage collagen degradation through specific inhibition of MMP-13. These MMP-13 inhibitors, as demonstrated with the inhibitor ALS 1-0635 described herein, are distinct from the broad-spectrum MMP inhibitors reported to date in terms of selectivity, mechanism of action, and musculoskeletal liability. This unique profile combined with their chondroprotective

ability, make this class of compounds suitable as potential disease-modifying OA drugs (DMOADs).

MATERIALS AND METHODS

Enzyme assays. MMP-13 activity assays were performed using the recombinant human MMP-13 catalytic domain (catalog no. 30100812; Invitek, Berlin, Germany) at a 1 n*M* concentration and the fluorogenic substrate MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 (catalog no. 444264; Calbiochem, Schallbach, Germany,) at a 5 μM concentration in 50 m*M* Tris (hydroxymethyl)aminomethane buffer (pH 7.5) containing 150 m*M* NaCl, 5 m*M* $CaCl_2$, and 0.05% Brij-35 (17). The assays were carried out in 96-well plates for 10 minutes at room temperature on a fluorescent plate reader, and the rate of substrate hydrolysis was determined by monitoring change in fluorescence at excitation and emission wavelengths of ~320 nm and 405 nm. The test compounds used for assessing the potency of inhibition of MMP-13 activity were dissolved in DMSO (final concentration 0.1%).

Selectivity assays were performed using catalytic domains of human MMPs and omniMMP fluorescent peptide MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2*AcOH (catalog no. SE-180; Biomol, Hamburg, Germany) as substrate (22). For MMP-3 and TACE assays, peptide substrates MCA-Arg-Pro-Lys-Pro-Val-Glu-NvaTrp-Arg-Lys(DNP)-NH_2 (catalog no. 480455; Calbiochem) and MCA-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 (catalog no. 616402; Calbiochem), respectively, were used. Aggrecanase assays were conducted by digesting the recombinant aggrecan interglobular domain and quantifying the cleaved aggrecan fragment with N-terminal ARGSVIL sequence, using the InviLISA kit (catalog no. 30510111; Invitek).

Enzyme kinetics and mechanism studies. To characterize MMP-13 inhibition kinetics of the test compounds, steady-state kinetics were assessed and Lineweaver-Burk plot analysis performed, using SigmaPlot Enzyme kinetic software. Competition experiments with acetohydroxamic acid (AcNHOH) and Dixon plot analysis of data were conducted to determine the binding mode of the inhibitors.

In vitro cartilage degradation assays. Freshly isolated bovine articular cartilage (~3-mm^2 pieces) or human cartilage samples cut into small pieces (obtained from OA patients undergoing knee joint replacement surgery) were incubated with 5 ng/ml interleukin-1α (IL-1α) plus 50 ng/ml oncostatin M (OSM) in the presence or absence of compounds, for 11 days (bovine cartilage) or 21 days (human cartilage). Culture medium was refreshed twice weekly. Collagen degradation products in the conditioned medium were measured using the C1,2C enzyme-linked immunosorbent assay (Ibex, Montreal, Quebec, Canada).

Animals. Rats were obtained from Harlan (Indianapolis, IN) and were housed 2 per cage in shoebox polycarbonate cages and maintained at the Alantos Pharmaceuticals animal facility. The animal facility was environmentally controlled to provide a temperature of 20–24°C, a relative humidity of 30–70%, and a 12-hour light/12-hour dark cycle. After acclimation for 3 days, the animals were identified by a unique line(s) or number on the tail, and randomized into treatment groups. Animals were allowed access ad libitum to standard rodent chow and fresh water. All animal procedures used in these studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Chemically induced OA. The rat model of monoiodoacetate (MIA)–induced OA (23–25) was used for assessing the effects of MMP-13 inhibitors on cartilage degradation and joint pain. Male Sprague-Dawley rats weighing 175–200 gm (12 per group) were anesthetized for 1 minute in an inhalation chamber, using a mixture of carbon dioxide and oxygen (60:40), and on day 0 were injected in the right knee joint with 50 μl saline containing 1 mg MIA. Control animals were injected in the right knee joint with only sterile saline (50 μl). Compound treatment was initiated 1 day before MIA injection (day −1). Test compounds or vehicle (0.5% methylcellulose) were administered twice daily for 14 days, by oral gavage. Pain was measured on day −1 and days 3, 7, and 14. Pain was assessed by placing the rats in a joint incapacitance tester (Columbus Instruments, Columbus, OH) and measuring the difference in hind paw weightbearing. Animals experiencing pain in one leg would be expected to shift their weight to the opposite (contralateral) leg. On day 14, the rats were killed and tibiae from the right joints were collected for assessing cartilage damage. The tibial cartilage surface was visually examined by 2 different observers under blinded conditions, and the degree of damage was scored on a scale of 0–4, with 0 representing no damage and 4 representing the most severe damage. The area of cartilage damage on the tibial surface was measured using a dissection microscope connected to a computer image capture and analysis system (Nikon NIS Elements; distributed by Micro Video Instruments, Avon, MA).

Surgical model of OA. The rat model of medial meniscal tear (26–28) was used to evaluate the chondroprotective ability of MMP-13 inhibitors in a chronic disease model of OA. These experiments were conducted at Bolder BioPath (Boulder, CO). Male Lewis rats (20 per group) weighing 270–302 gm were anesthetized with isoflurane and prepared for surgery. A skin incision was made over the medial aspect of the right knee joint, and the medial collateral ligament was exposed by blunt dissection. Then the meniscus was cut through the full thickness to simulate a complete tear. Skin and subcutis were closed with 4-0 Vicryl suture. Twice-daily oral dosing (12-hour intervals) with vehicle (0.5% methylcellulose with 2% Tween 80) or MMP-13 inhibitors (1, 10, 30, or 60 mg/kg) was initiated the day before surgery and continued for 21 days after surgery. The animals were bled via the tail vein for collection of plasma on day −1 and day 20. Animals were killed, and right knees collected for histopathologic evaluation of chondroprotective effects. All analyses were done without knowledge of treatment group identity.

Cartilage degradation across the medial tibial plateau was scored by subdividing the tibial plateau into 3 zones and scoring each zone for chondrocyte death/loss, proteoglycan loss, and collagen loss or fibrillation, using a scale of 0–5, with 0 representing no abnormality and 5 representing the most severe abnormality. Cartilage degeneration width (μm) was a measurement of tibial cartilage degeneration extending through >50% of the cartilage thickness. Depth of cartilage degradation was expressed as the ratio of depth of damaged area:depth to tidemark. Osteophyte scores were assigned to the largest osteophyte in each section, according to the following criteria: 1 = ≤299 μm, 2 = 300–399 μm, 3 = 400–499 μm,

Table 1. Potency and selectivity of MMP-13 inhibitors*

Inhibitor	IC_{50}, n*M*										
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-12	MMP-13†	MMP-14	TACE	Aggrecanase 1
ALS 1-0635	>20,000	>20,000	18,000	>20,000	>20,000	>20,000	>20,000	3.6 ± 0.1	>20,000	>20,000	>20,000
ALS 1-0892	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	8,500	0.7 ± 0.2	>20,000	24,000	>20,000
ALS 1-1069	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	1.3 ± 0.4	>20,000	>20,000	>20,000
ALS 1-1084	>20,000	>20,000	16,000	>20,000	>20,000	>20,000	14,406	0.9 ± 0.3	>20,000	>20,000	>20,000
ALS 1-1087	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	2.8 ± 0.8	>20,000	>20,000	>20,000
ALS 1-0935	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	0.9 ± 0.0	>20,000	>20,000	>20,000
ALS 1-1089	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	0.8 ± 0.2	>20,000	>20,000	>20,000
ALS 1-1124	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	13,785	0.2 ± 0.0	>20,000	>20,000	>20,000
Ilomastat†	1.4 ± 0.1	0.6 ± 0.01	6.8 ± 0.5	NT	0.4 ± 0.02	0.5 ± 0.1	1.1 ± 0.2	0.4 ± 0.02	2.5 ± 0.3	45 ± 4	547 ± 147

* Enzyme assays were performed as described in Materials and Methods, using catalytic domains of human matrix metalloproteinases (MMPs) and fluorogenic substrates. Aggrecanase assays were performed using aggrecan interglobular domain, and the degradation products quantified by enzyme-linked immunosorbent assay. Fifty percent inhibition concentration (IC_{50}) determinations were based on evaluation of 8 different concentrations of test compounds (0–20,000 n*M*), with each concentration tested in duplicate. Ilomastat, a broad-spectrum MMP inhibitor, was included as a positive control. NT = not tested.
† Mean ± SD.

and 4 = ≥500 μm. Bone scores were obtained by scoring the calcified cartilage layer and subchondral bone increase in basophilia, fragmentation of tidemark, and marrow changes, using a score of 0–5, with 5 representing the greatest severity.

Rat model of musculoskeletal side effects. The rat model of MSS was used to assess whether MMP-13 inhibitors have the joint toxicity associated with the broad-spectrum MMP inhibitors (8). These experiments were conducted at Seventh Wave Laboratories (Chesterfield, MO). Male Sprague-Dawley IGS rats (Charles River, Wilmington, MA) (202–239 gm; 6 rats per group) were dosed orally with vehicle (aqueous 10% hydroxypropyl-β-cyclodextrin, 50% polyethylene glycol 400, 5% DMSO) or with 100 mg/kg/day MMP-13 inhibitor, or intraperitoneally with 6.25 mg/kg/day Marimastat administered via Alzet pump. The animals were treated daily for 14 days and observed for clinical signs of MSS. Blood samples were collected on days 1 and 14 for determining plasma drug concentrations. Joints were collected when animals were killed, and were evaluated for histologic changes indicative of MSS. Gross postmortem examination was also conducted, and the heart, kidney, liver, spleen, and hind limbs were collected for gross and microscopic pathologic evaluation.

RESULTS

Discovery and characterization of MMP-13 inhibitors. A diverse variety of non–hydroxamic acid–based compounds were synthesized, with the aim of discovering non–zinc-binding inhibitors with nanomolar potency against MMP-13 activity and high selectivity against other MMPs. Refinement of structure, based on molecular modeling and MMP-13 x-ray crystal data, and screening of compounds against human MMP-13 catalytic domain resulted in identification of several different classes of compounds with picomolar potency (50% inhibition concentration [IC_{50}] 0.2–3.6 n*M*) (Table 1). Selectivity assays against catalytic domains of MMPs, TACE, and aggrecanase revealed compounds with >20,000 n*M* selectivity (Table 1).

Steady-state kinetics experiments and Lineweaver-Burk plot analysis of rate versus substrate concentration with the representative MMP-13 inhibitor ALS 1-0635 indicated linear, noncompetitive (mixed) inhibition, with a K_i of 0.78 n*M*, a K_m of 5.5 μ*M*, and a maximum volume of 430 pmoles/minute^{-1} (data not shown). Dixon plot analysis of data from competition studies with the zinc chelator AcNHOH and the MMP-13 inhibitor ALS 1-0635 demonstrated nonexclusive binding (data not shown), indicating that ALS 1-0635 does not compete with AcNHOH for catalytic zinc. Studies with other representative MMP-13 inhibitors also demonstrated noncompetitive inhibition and nonexclusive binding with AcNHOH (data not shown). In contrast, Ilomastat, a standard broad-spectrum inhibitor, showed no selectivity against any of the enzymes tested (Table 1) and was found to compete with AcNHOH for catalytic zinc (data not shown).

Protection against IL-1α– and OSM-induced cartilage degradation. The chondroprotective ability of MMP-13 inhibitors was assessed in vitro by measuring release of C1,2C, a specific marker of collagenase-mediated collagen loss, from IL-1α– and OSM-stimulated bovine articular cartilage or surgical samples of human OA cartilage. The MMP-13 inhibitor ALS 1-0635 inhibited bovine articular cartilage degradation in a dose-dependent manner (48.7% and 87.1% inhibition at 500 n*M* and 5,000 n*M*, respectively) (Figure 1A). The broad-spectrum inhibitor Ilomastat at 50 n*M* inhibited C1,2C release by 96.8%. Similarly, ALS 1-0635 was

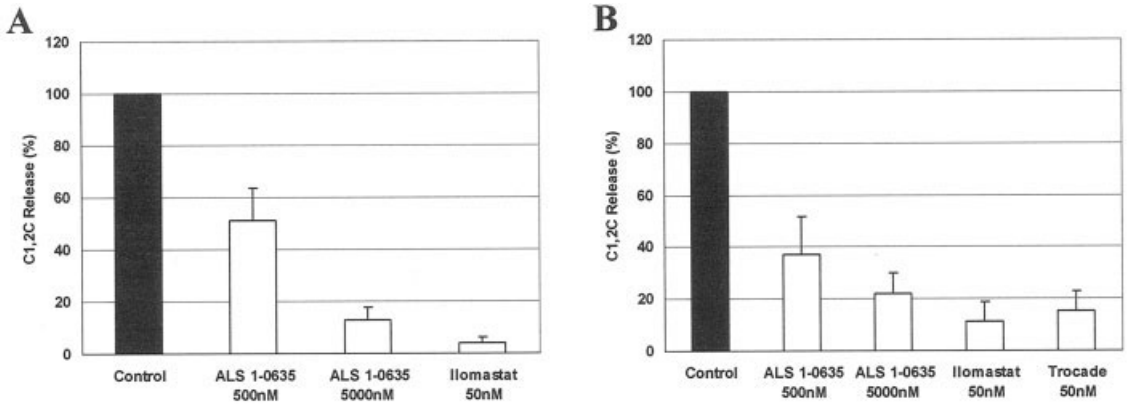

Figure 1. Protection against interleukin-1 (IL-1)– and oncostatin M (OSM)–induced degradation of articular cartilage by matrix metalloproteinase 13 (MMP-13) inhibition. Bovine articular cartilage cultures (**A**) or human knee osteoarthritis (OA) cartilage cultures (**B**) were incubated with 5 ng/ml IL-1α and 50 ng/ml OSM in the absence (control) or presence of the MMP-13 inhibitor ALS 1-0635. The incubation period was 11 days and 21 days, respectively, in studies of bovine articular cartilage and studies of human OA cartilage. Incubation with Ilomastat, a broad-spectrum MMP inhibitor, was included as a positive control in bovine cartilage studies and human OA cartilage studies; tromade, another broad-spectrum MMP inhibitor, was also included as a positive control in human OA cartilage studies. Collagen degradation products in supernatants of the explants were detected by enzyme-linked immunosorbent assay measurement of C1,2C release, expressed as the percentage of C1,2C release in control cultures. Values are the mean and SEM (n = 5 in **A**; n = 8 in **B**).

effective in inhibiting IL-1α– and OSM-induced C1,2C loss from human OA cartilage cultures (62.8% at 500 n*M* and 78.3% at 5,000 n*M*) (Figure 1B). The broad-spectrum MMP inhibitors Ilomastat and Trocade at 50 n*M* inhibited IL-1α– and OSM-induced C1,2C release by 88.6% and 84.9%, respectively (Figure 1B).

Modulation of cartilage damage and joint pain in the rat model of MIA-induced OA. We evaluated the effects of ALS 1-0635 (30 mg/kg administered twice daily) in a model of cartilage damage and joint pain induced by a single intraarticular injection of MIA into rat knee joints. Visual examination of the gross morphology of the tibial cartilage surface of the right knee joint from saline-treated control animals revealed an intact smooth articular surface, whereas the tibial plateau of MIA-treated animals exhibited extensive damage, characterized by pitting of the articular surface. In MIA-injected animals that were treated with ALS 1-0635, the mean ± SEM cartilage damage score was significantly lower than that in vehicle-treated animals (1.3 ± 0.3 versus 2.2 ± 0.4; $P \leq 0.05$). Cartilage damage in MIA-injected rats affected 50–100% of the surface area, with signs of subchondral bone exposure. In MIA-injected animals treated with compound, in contrast, the cartilage damage was markedly less extensive (≤50% of surface area). The mean ± SEM area of cartilage damage in the ALS 1-0635–treated rats was 4.0 ± 1.3 mm^2, compared with 7.7 ± 2.0 mm^2 in vehicle-treated animals.

ALS 1-0635–mediated reductions in both the cartilage damage score and the cartilage damage area were statistically significant (41% and 48%, respectively, compared with findings in rats treated with MIA alone; $P \leq 0.05$). None of the saline-injected control animals exhibited any cartilage damage.

ALS 1-0635 also appeared to modulate joint pain. ALS 1-0635–treated animals showed a 20%, 10%, and 24% improvement in ability to bear weight on their right knee joint on days 3, 7, and 14, respectively, compared with animals treated with MIA alone ($P \leq 0.05$). As expected, saline-treated control animals were able to bear weight equally on both hind limbs throughout the study. The compound was well tolerated and showed no adverse effects in this study. Additional details on the findings in rats with MIA-induced OA are available upon request from the corresponding author.

Efficacy in the surgical model of OA. Rats with medial meniscal tear–induced cartilage degeneration in the right knee joint were treated twice daily by oral gavage, beginning 1 day prior to surgery and continuing for 3 weeks after surgery, with vehicle or ALS 1-0635 (1, 10, 30, or 60 mg/kg). Right knees were evaluated histologically for chondroprotective effects. More than 40% of rats subjected to medial meniscal tear and treated with vehicle exhibited moderate to severe degenerative changes in their right knee joint, characterized by chondrocyte and matrix loss, fibrillation, osteophyte formation, and chondrocyte cloning (Figure 2A). The medial

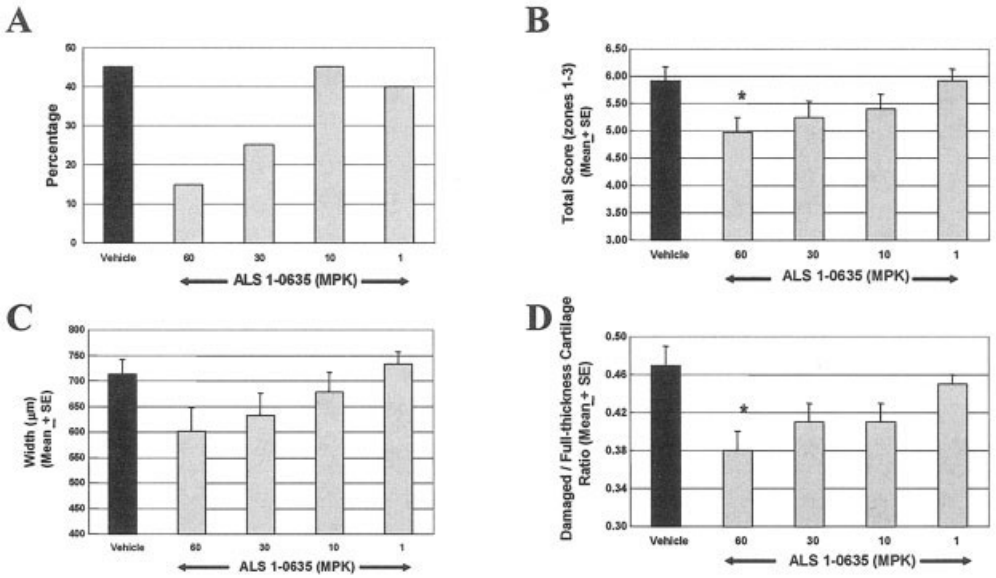

Figure 2. Effect of MMP-13 inhibition on cartilage degradation in the rat medial meniscal tear model of OA. **A,** Percentage of animals in each treatment group with a total cartilage degradation score of >6 (sum of scores in 3 zones, each scored on scale of 0–5 [maximum possible total score 15]). **B,** Total cartilage degradation score (zones 1–3) in the tibial plateau. **C,** Width of the area of tibial cartilage degeneration extending through >50% of the cartilage thickness. **D,** Depth of cartilage degradation, expressed as the ratio of the depth of the damaged area:depth to the tidemark. Twenty rats were included in each treatment group. $* = P \leq 0.05$ versus all other treatment groups, by two-way analysis of variance with Fisher's least significant difference test for pairwise multiple comparisons. MPK = mg/kg (see Figure 1 for other definitions).

tibial plateau, especially the outer two-thirds, was affected the most, as demonstrated by the higher scores for cartilage degeneration (mean ± SEM 5.92 ± 0.29), width of the area of cartilage damage (713.3 ± 28.6 μm), and ratio of the depth of damaged cartilage to the full thickness of cartilage (0.47 ± 0.02) (Figures 2B–D). Substantial changes in bone, as indicated by the bone score (mean ± SEM 2.95 ± 0.18) and osteophyte score (3.62 ± 0.15), were also seen in the medial tibia, subjacent to the areas of greatest lesion severity (Figures 3A and B).

Administration of ALS 1-0635 (1–60 mg/kg twice daily) resulted in beneficial, dose-responsive effects on the majority of the parameters measured, with no ad-

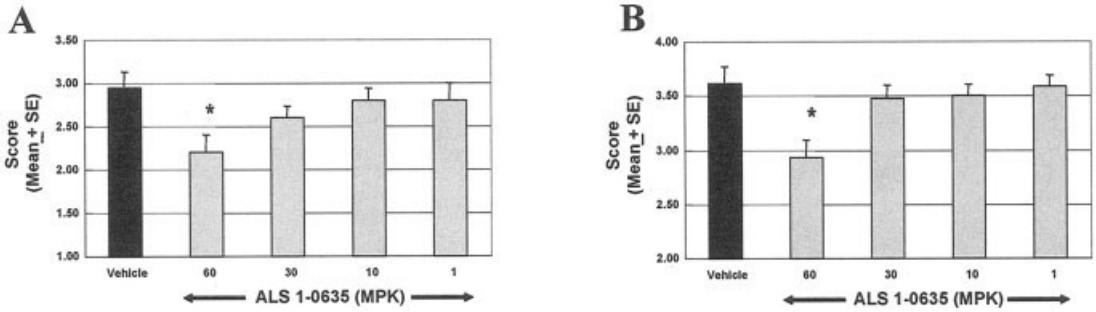

Figure 3. Effect of MMP-13 inhibition on bone and osteophyte scores in the rat medial meniscal tear model of OA. **A,** Bone scores, obtained by scoring of histologic changes in the calcified cartilage layer and subchondral bone on a scale of 0–5 (5 indicating the greatest severity). **B,** Osteophyte scores, obtained by scoring of the largest osteophyte in each section on a scale of 1–4 (1 = ≤299 μm; 2 = 300–399 μm; 3 = 400–499 μm; 4 = ≥500 μm). Twenty rats were included in each treatment group. $* = P \leq 0.05$ versus all other treatment groups, by two-way analysis of variance with Fisher's least significant difference test for pairwise multiple comparisons. MPK = mg/kg (see Figure 1 for other definitions).

Table 2. Comparison of plasma drug concentrations and incidence of musculoskeletal syndrome in rats treated with ALS 1-0635 or Marimastat*

	ALS 1-0635 (n = 6)	Marimastat (n = 6)
Inhibitor type	MMP-13–specific	Broad-spectrum
Dose, mg/kg/day	100	6.25
Day-14 AUC_{0-24}, mg/hour/ml	743	ND
No. of rats with MSS-like lesions	0/6	6/6

* Sprague-Dawley rats were dosed orally with ALS 1-0635 or intraperitoneally via Alzet minipump with Marimastat for 14 days and observed for clinical signs of musculoskeletal syndrome (MSS). Plasma samples were analyzed for drug concentrations. AUC_{0-24} = 0–24-hour area under the curve; ND = not detected.

verse effects on weight gain or growth plate morphology. ALS 1-0635 treatment resulted in a marked reduction in the percentage of rats with moderate to severe cartilage degradation (reductions of ~67% and ~44% at 60 mg/kg and 30 mg/kg, respectively) (Figure 2A). The ALS 1-0635–treated rats also had reduced scores for cartilage degeneration (Figure 2B). The effect of the compound was more pronounced in the inner one-third of the tibial plateau (89%, 78%, 89%, and 33% at the 60, 30, 10, and 1 mg/kg doses, respectively). The treatment also reduced the width of the area of cartilage damage and the ratio of the depth of damaged cartilage to the full thickness of cartilage (Figures 2C and D). The beneficial effects were also reflected in bone and osteophyte scores (Figures 3A and B).

Studies of preclinical MSS. The potential induction of MSS-like symptoms by the MMP-13–specific inhibitor ALS 1-0635 was assessed in the rat model of MSS. Animals treated with ALS 1-0635 (100 mg/kg/day) were observed for clinical signs and histologic changes indicative of MSS, and the findings were compared with those in animals treated with Marimastat, which is known to induce MSS in humans and has been shown to induce MSS-like changes in rats and monkeys (8) (Table 2).

Clinical assessment on the day before the animals were killed revealed grossly evident joint swelling and reduced range of motion of the hind limbs in all rats in the Marimastat-treated group. However, all ALS 1-0635–treated or vehicle-treated animals appeared normal. Microscopic histopathologic changes were also observed only in the Marimastat-treated group. Animals in this group had dysplasia of the growth plate cartilage, resulting in widening of cartilage and frequent subchondral fracture. In addition, they exhibited intra- and periarticular fibroplasia and mild to moderate intraarticular inflammation (subacute to acute). None of the animals in the vehicle or ALS 1-0635 group showed any treatment-related changes in the joint. Postmortem examination and gross and microscopic evaluation revealed treatment-related effects such as discoloration and fibrosis of various organs, including kidney, liver, and spleen, in the Marimastat-treated rats. No such changes were observed in animals from the vehicle- or ALS 1-0635–treated groups. Toxicokinetic analysis demonstrated the presence of parent compound in the plasma of ALS 1-0635–treated animals. The exposure on day 14 in the combined group of animals treated with ALS 1-0635 at100 mg/kg/day was 743 μg/hour/ml (0–24-hour area under the curve). Such analyses were not performed in samples from Marimastat-treated animals since the toxicity findings were consistent with those reported in the literature (8).

DISCUSSION

Over the last 2 decades, data implicating MMPs in the pathogenesis of human OA and preclinical demonstration of the success of broad-spectrum MMP inhibitors led to development of several hydroxamic acid– or carboxylic acid–based inhibitors as potential DMOADs. However, clinical utility of these broad-spectrum MMP inhibitors has been limited by debilitating, dose- and duration-dependent MSS (fibroplasia or tendinitis) in humans (6,7). Although no specific MMP has been implicated, it is believed that nonselective inhibition of multiple MMPs is the primary reason for the observed toxicity (8). Therefore, our strategy for development of DMOADs was to focus on identification of highly selective allosteric MMP-13 inhibitors free of hydroxamic acid or other zinc-chelating functional groups that contribute to inhibition of multiple MMPs. The rationale for targeting MMP-13 came from 1) overwhelming data on a potential role of MMP-13 in the pathogenesis of OA (11–21), and 2) data from studies of MMP-13 knockout mice and humans with an MMP-13 mutation, suggesting lack of MSS liability (29–32).

Identification and optimization of allosteric binding, non–hydroxamate-based compounds and refinement of structure based on structure–activity relationship, molecular modeling, and MMP-13 x-ray crystal data led to discovery of several different classes of highly potent (IC_{50} 0.2–3.6 n*M*) MMP-13 inhibitors (Table 1). Evaluation of these compounds in selectivity assays against various MMPs (MMP-1, 2, 3, 7, 8, 9, 12, and 14) and other related zinc metalloproteinases including TACE and aggrecanase revealed >20,000 n*M* selectivity

(Table 1). In contrast, selectivity data on Ilomastat (Table 1) and other broad-spectrum inhibitors reported in the literature indicate that, although potent against MMP-13 activity, the broad-spectrum inhibitors lack selectivity (3,5,33,34). The only other MMP inhibitors with selectivity comparable with that of the MMP-13 inhibitors in the present study are the class of MMP-13 inhibitors recently reported in the literature (21). Steady-state kinetics and Lineweaver-Burk plot analysis of data on our MMP-13 inhibitor ALS 1-0635 demonstrated noncompetitive (mixed) inhibition.

To obtain additional information on the binding characteristics of MMP-13 inhibitors, competition experiments with ALS 1-0635 and the zinc chelator AcNHOH were performed. Dixon plot analysis of the data demonstrated nonexclusive binding (data not shown), suggesting that ALS 1-0635 does not compete with AcNHOH for active-site zinc. Enzyme inhibitor preincubation dilution studies indicated that MMP-13 inhibition of ALS 1-0635 is reversible. Steady-state kinetic competition experiments performed with our other proprietary MMP-13 inhibitor series also demonstrated noncompetitive inhibition and lack of chelation of catalytic zinc.

These efforts resulted in the discovery of novel classes of highly potent, noncompetitive MMP-13–selective inhibitors with a different binding mode than that of broad-spectrum MMP inhibitors. Molecular modeling data suggest that these compounds bind to the S1' specificity loop of MMP-13, similar to the MMP-13–selective inhibitors described recently (21). This could account for the high potency and selectivity of the MMP-13 inhibitors. Furthermore, the MMP-13 inhibitors described in this report do not chelate active-site zinc, and as a consequence would not be expected to inhibit other zinc metalloproteinases, as observed by the extremely high selectivity. In contrast, almost all of the broad-spectrum and partially selective MMP inhibitors described to date possess a hydroxamic acid–, carboxylic acid–, or other metal-chelating group that binds to the catalytic zinc ion of MMPs and competes with substrate binding.

To test whether MMP-13–selective inhibition is sufficient to inhibit collagen degradation in a physiologic setting, compounds were evaluated in normal bovine articular cartilage and human OA cartilage cultures that were stimulated with IL-1 and OSM to degrade collagen. The dose-dependent inhibition of C1,2C release by ALS 1-0635 in both the bovine cartilage cultures (48.7% and 87.1% at 500 and 5,000 n*M*, respectively) and the human cartilage cultures (62.8% and 78.3% at 500 and 5,000 n*M*, respectively) (Figures 1A and B) demonstrates that MMP-13 inhibition is sufficient to inhibit collagen degradation in a physiologic system. These results are consistent with the IC_{50} reported for the MMP-13–selective inhibitors in bovine nasal cartilage cultures (mean ± SD 1.3 ± 0.4 μM) (21). Higher levels of inhibition seen with broad-spectrum MMP inhibitors (Figures 1A and B) suggest that other MMPs, such as MMP-1, may also contribute to the collagen degradation process. However, data from screening of our other MMP-13 inhibitors in the bovine cartilage cultures demonstrated complete inhibition at doses comparable with or 10-fold lower than those of the broad-spectrum inhibitors used in the present study (data not shown). These findings suggest that MMP-13 inhibition alone may be sufficient to block collagen degradation, at least under in vitro conditions.

Taken together, our results clearly demonstrate that ALS 1-0635 not only has excellent oral bioavailability and systemic exposure (data available upon request from the corresponding author), but also distributes to the synovial compartment and is able to penetrate cartilage effectively. These findings represent unique features and significant improvements over the broad-spectrum hydroxamic acid– and carboxylic acid–containing MMP inhibitors, which have been problematic in terms of pharmacokinetics and cartilage penetration.

Compared with plasma concentrations of ALS 1-0635 (28,088 ng/ml, or 60.73 μM), significantly lower concentrations have been reported for the carboxylic acid–based MMP inhibitors PGE-6292544 (~5,000 ng/ml) and PGE-2909492 (500 ng/ml) and for the hydroxamate-based MMP inhibitor PGE-3321996 (~40 ng/ml) (35). Similar to the protocol used in the present study, these broad-spectrum MMP inhibitors were administered orally to rats (25 mg/kg twice daily), and plasma concentrations were measured 2 hours after the last dosing (concentrations at 2 hours posttreatment extrapolated from a graph included in the report [35]). Interestingly, neither of the carboxylic acid–based inhibitors was detectable in cartilage, whereas the cartilage concentration of the hydroxamic acid compound was 282 n*M* (C_{av}) (35). In terms of both oral availability and exposure of target tissues, the MMP-13 inhibitors in the present study have a much better pharmacokinetic profile than the broad-spectrum inhibitors reported previously.

To determine whether MMP-13 inhibition leads to chondroprotection in a chronic disease setting, ALS 1-0635 was evaluated in 2 different models in rats, i.e., MIA-induced and surgically induced OA. Based on

scoring by 2 observers under blinded conditions, ALS 1-0635 was effective in significantly reducing cartilage damage (41%) as well as actual area of cartilage damage (48%) ($P < 0.05$) (detailed data available upon request from the corresponding author). Furthermore, ALS 1-0635 treatment significantly ($P < 0.05$) blocked joint pain, as assessed by weightbearing on the hind limbs (detailed data available upon request from the corresponding author). Joint pain is a cardinal sign of human OA, and reduction in pain along with prevention of further tissue damage and maintaining function are principal treatment goals. Evaluation of several broad-spectrum inhibitors (PGE-621143, PGE-3162689, and PGE-6912923) in the MIA model showed chondroprotection (36–42% inhibition) comparable with that obtained with ALS 1-0635 (36).

Interestingly, carboxylic acid–based inhibitors that exhibited good systemic exposure but were not detectable in cartilage were inactive in the MIA model, and the hydroxamic acid compound, with a cartilage concentration of 282 n*M* (C_{av}), inhibited 28% of MIA-induced cartilage degradation (35). These findings further highlight the importance of studying cartilage drug concentrations in relation to plasma exposure.

ALS 1-0635 treatment of rats subjected to medial meniscal tear resulted in a dose-dependent reduction in cartilage and bone degradation scores ($P < 0.05$) (Figure 3A). The treatment also reduced the osteophyte score (Figure 3B) and measurement (data not shown). The beneficial effects were consistently significant at the 60 mg/kg dose ($P < 0.05$) (Figures 2 and 3). There were no adverse effects on weight gain or growth plate morphology.

Previously reported experiments conducted with a broad-spectrum hydroxamate-based inhibitor in the rat medial meniscal tear model demonstrated 14–50% inhibition of tibial cartilage degradation and 18–50% reduction of osteophyte scores (27). ALS 1-0635, used in the present study, resulted in 16% and 19% reductions in tibial and osteophyte scores, respectively. It is not clear whether these differences reflect differences in the scoring methods used in the two studies, or whether they indicate that the broad-spectrum MMP inhibitor was more efficacious. However, the data on MMP-13–selective inhibitors in the various models in the present study, as well as those reported recently by another group studying both rat and rabbit models (21), clearly demonstrate that MMP-13 inhibition is sufficient for inhibiting degradative osteoarthritic-like changes. It is possible that inhibition of multiple MMPs may lead to relatively higher efficacy. However, utility of broad-spectrum MMP inhibitors for treatment of OA is limited by MSS liability (37). Therefore, we included assessment of MSS liability as a critical component of our effort to develop MMP-13 inhibitors for OA.

Experiments using the rat model of MSS showed that after 2 weeks of treatment, none of the animals receiving ALS 1-0635 at 100 mg/kg/day exhibited any adverse clinical signs or histologic changes characteristic of MSS (detailed results available upon request from the corresponding author). In contrast, all 6 animals treated with Marimastat (6.25 mg/kg/day) showed clinical as well as histologic signs of MSS. Plasma concentrations of Marimastat were not measured in the present study. The average plasma concentrations of ALS 1-0635 on day 1 and day 14 were 2,000 and 235 ng/ml, respectively. These concentrations are, respectively, 1,000-fold and 100-fold higher than the in vitro IC_{50} of ALS 1-0635 against MMP-13. Plasma Marimastat concentrations (trough) of 10 ng/ml are known to induce MSS in humans (38). Considering that the IC_{50} of Marimastat against MMP-13 is 4.59 n*M*, the margin between potency and toxicity is only ~2-fold. Marimastat also has nanomolar potency against other MMPs, with an IC_{50} of 1.94 n*M* and 2.10 n*M* against collagenase 1 (MMP-1) and collagenase 2 (MMP-8), respectively (39).

Thus, ALS 1-0635 showed no signs of inducing MSS even at concentrations 200-fold higher than the concentrations of Marimastat known to induce this condition. Recent reports on MMP-13–selective inhibitors also indicate that MMP-13 inhibition does not induce MSS in the rat model (21). These findings, together with results of studies of MMP-13–knockout mice and MMP-13 mutations in humans, provide ample evidence for a lack of association of MSS with MMP-13 inhibition.

In conclusion, the compounds described herein represent a novel class of MMP-13 inhibitors. They are mechanistically distinct from previously reported broad-spectrum MMP inhibitors and do not present the problems associated with these inhibitors, including selectivity, poor pharmacokinetics, and MSS liabilities. Furthermore, as demonstrated with ALS 1-0635, the MMP-13 inhibitors are chondroprotective and can potentially modulate joint pain, and thus are uniquely suited as potential DMOADs.

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AUTHOR CONTRIBUTIONS

Dr. Powers had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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